DNA MOLECULES ENCODING BACTERIAL LYSINE 2,3-AMINOMUTASE

FIELD OF THE INVENTION

The present invention relates to DNA molecules that encode lysine 2,3-aminomutase. More particularly, this invention relates to the use of recombinant host cells comprising such DNA molecules to produce pure L-β-lysine.

BACKGROUND OF THE INVENTION

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government Funds, specifically NIH Grant Nos. DK28607; DK09306; GM31343; GM30480; GM10816; GM14401; GM15395; GM51806, and GM18282. Therefore, the U.S. Government has certain rights in this invention.

RELATED ART

Although less abundant than the corresponding α-amino acids, β-amino acids occur in nature in both free forms and in peptides. Cardillo and Tomasini, *Chem. Soc. Rev. 25*:77 (1996); Sewald, *Amino Acids 11*:397 (1996). Since β-amino acids are stronger bases and weaker acids than α-amino acid counterparts, peptides that contain a β-amino acid in place of an α-amino acid, have a different skeleton atom pattern, resulting in new properties. For example, various peptides are protease inhibitors because the presence of the β-amino-α-hydroxy acid motif acts as a transition state mimic of peptide hydrolysis.

β-Amino acids are of particular interest in the preparation of medicaments, such as β-lactams. Well-known β-lactam antimicrobial agents include penicillins, cephalosporins, carbapenems, and monobactams. Other examples of medically useful molecules that contain β-amino-α-hydroxy acids include the anti-tumor agent taxol, the anti-bacterial agent, dideoxykanamicin A, bestatin, an

immunological response modifier, the kynostatins, which are highly potent human immunodeficiency virus-1 protease inhibitors, and microginin, a tetrapeptide which has anti-hypertensive properties. Accordingly, enantiomerically pure β-amino-α-hydroxy acids are of considerable importance as crucial components of pharmacologically active compounds.

In the 1950's, L-β-lysine was identified in several strongly basic peptide antibiotics produced by *Streptomyces*. Antibiotics that yield L-β-lysine upon hydrolysis include viomycin, streptolin A, streptothricin, roseothricin and geomycin. Stadtman, *Adv. Enzymol. Relat. Areas Molec. Biol. 38*:413 (1973). β-Lysine is also a constituent of antibiotics produced by the fungi *Nocardia*, such as mycomycin, and β-lysine may be used to prepare other biologically active compounds. However, the chemical synthesis of β-lysine is time consuming, requires expensive starting materials, and results in a racemic mixture.

Therefore, a need exists for an improved method of preparing enantiomerically pure β-amino acids, such as β-lysine.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides an isolated DNA molecule comprising a nucleotide sequence that encodes lysine 2,3-aminomutase.

In another aspect, the present invention provides an expression vector comprising an isolated DNA molecule having a nucleotide sequence that encodes lysine 2,3-aminomutase.

The present invention additionally provides a method of producing lysine 2,3-aminomutase comprising the steps of culturing a host cell containing an expression vector having a nucleotide sequence that encodes lysine 2,3-aminomutase and isolating lysine 2,3-aminomutase from the cultured host cells.

The present invention provides, in a further aspect, a method of producing L-β-lysine from L-lysine comprising incubating L-lysine in a solution containing purified lysine 2,3-aminomutase and isolating the L-β-lysine from the solution.

Still another aspect of the present invention is a method of producing L-β-lysine from L-lysine comprising the steps of incubating culturing a host cell in the

presence of L-lysine, wherein the cultured host cell expresses lysine 2,3-aminomutase and isolating the L-β-lysine from the cultured host cell.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions.

In the description that follows, a number of terms are utilized extensively. Definitions are herein provided to facilitate understanding of the invention.

Structural gene. A DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide (protein).

Promoter. A DNA sequence which directs the transcription of a structural gene to produce mRNA. Typically, a promoter is located in the 5′ region of a gene, proximal to the start codon of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Enhancer. A promoter element. An enhancer can increase the efficiency with which a particular gene is transcribed into mRNA irrespective of the distance or orientation of the enhancer relative to the start site of transcription.

Complementary DNA (cDNA). Complementary DNA is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule derived from a single mRNA molecule.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Cloning vector. A DNA molecule, such as a plasmid, cosmid, phagemid, or bacteriophage, which has the capability of replicating autonomously in a host cell and which is used to transform cells for gene manipulation. Cloning vectors

typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences may be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene which is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

Expression vector. A DNA molecule comprising a cloned structural gene encoding a foreign protein which provides the expression of the foreign protein in a recombinant host. Typically, the expression of the cloned gene is placed under the control of (*i.e.,* operably linked to) certain regulatory sequences such as promoter and enhancer sequences. Promoter sequences may be either constitutive or inducible.

Recombinant host. A recombinant host may be any prokaryotic or eukaryotic cell which contains either a cloning vector or expression vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell. For examples of suitable hosts, see Sambrook *et al.,* MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989) ["Sambrook"].

As used herein, a **substantially pure protein** means that the desired purified protein is essentially free from contaminating cellular components, as evidenced by a single band following polyacrylamide-sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). The term "substantially pure" is further meant to describe a molecule which is homogeneous by one or more purity or homogeneity characteristics used by those of skill in the art. For example, a substantially pure lysine 2,3-aminomutase will show constant and reproducible characteristics within standard experimental deviations for parameters such as the following: molecular weight, chromatographic migration, amino acid composition, amino acid sequence, blocked or unblocked N-terminus, HPLC elution profile, biological activity, and other such parameters. The term, however, is not meant to exclude artificial or synthetic mixtures of lysine 2,3-aminomutase with other compounds. In addition, the term is not meant to exclude lysine 2,3-aminomutase fusion proteins isolated from a recombinant host.

2. Isolation of a DNA Molecule That Encodes the *Clostridium* Lysine 2,3-Aminomutase

Lysine 2,3-aminomutase catalyzes the reversible isomerization of L-lysine into L-β-lysine. The enzyme isolated from *Clostridium subterminale* strain SB4 is a hexameric protein of apparently identical subunits, which has a molecular weight of 259,000, as determined from diffusion and sedimentation coefficients. Chirpich *et al., J. Biol. Chem. 245*:1778 (1970); Aberhart *et al., J. Am. Chem. Soc. 105*:5461 (1983); Chang *et al., Biochemistry 35*:11081 (1996). The clostridial enzyme contains iron-sulfur clusters, cobalt and zinc, and pyridoxal 5'-phosphate, and it is activated by *S*-adenosylmethionine. Unlike typical adenosylcobalamin-dependent aminomutases, the clostridial enzyme does not contain or require any species of vitamin B_{12} coenzyme.

Although the existence of the clostridial lysine 2,3-aminomutase has been known for over 25 years, there is no report in the scientific literature on the isolation of the gene encoding the enzyme. As described herein, however, DNA molecules encoding the clostridial lysine 2,3-aminomutase gene now have been isolated from a genomic library made from the DNA of *Clostridium subterminale* strain SB4. The nucleotide and predicted amino acid sequences of clostridial lysine 2,3-aminomutase (SEQ ID NOs:1 and 2) are:

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1   ATGATAAATA GAAGATATGA ATTATTTAAA GATGTTAGCG ATGCAGACTG
51  GAATGACTGG AGATGGCAAG TAAGAAACAG AATAGAAACT GTTGAAGAAC
101 TAAAGAAATA CATACCATTA ACAAAAGAAG AAGAAGAAGG AGTAGCTCAA
151 TGTGTAAAAT CATTAAGAAT GGCTATTACT CCATATTATC TATCATTAAT
201 CGATCCTAAC GATCCTAATG ATCCAGTAAG AAAACAAGCT ATTCCAACAG
251 CATTAGAGCT TAACAAAGCT GCTGCAGATC TTGAAGACCC ATTACATGAA
301 GATACAGATT CACCAGTACC TGGATTAACT CACAGATATC CAGATAGAGT
351 ATTATTATTA ATAACTGATA TGTGCTCAAT GTACTGCAGA CACTGTACAA
401 GAAGAAGATT TGCAGGACAA AGCGATGACT CTATGCCAAT GGAAAGAATA
451 GATAAAGCTA TAGATTATAT CAGAAATACT CCTCAAGTTA GAGACGTATT
501 ATTATCAGGT GGAGACGCTC TTTTAGTATC TGATGAAACA TTAGAATACA
551 TCATAGCTAA ATTAAGAGAA ATACCACACG TTGAAATAGT AAGAATAGGT
601 TCAAGAACTC CAGTTGTTCT TCCACAAAGA ATAACTCCAG AACTTGTAAA
651 TATGCTTAAA AAATATCATC CAGTATGGTT AAACACTCAC TTTAACCATC
701 CAAATGAAAT AACAGAAGAA TCAACTAGAG CTTGTCAATT ACTTGCTGAC
751 GCAGGAGTAC CTCTAGGAAA CCAATCAGTT TTATTAAGAG GAGTTAACGA
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801 TTGCGTACAC GTAATGAAAG AATTAGTTAA CAAATTAGTA AAAATAAGAG
851 TAAGACCTTA CTACATCTAT CAATGTGACT TATCATTAGG ACTTGAGCAC
901 TTCAGAACTC CAGTTTCTAA AGGTATCGAA ATCATTGAAG GATTAAGAGG
951 ACATACTTCA GGATACTGCG TACCAACATT CGTTGTTGAC GCTCCAGGTG
1001 GTGGTGGAAA AACACCAGTT ATGCCAAACT ACGTTATTTC ACAAAGTCAT
1051 GACAAAGTAA TATTAAGAAA CTTTGAAGGT GTTATAACAA CTTATTCAGA
1101 ACCAATAAAC TATACTCCAG GATGCAACTG TGATGTTTGC ACTGGCAAGA
1151 AAAAAGTTCA TAAGGTTGGA GTTGCTGGAT TATTAAACGG AGAAGGAATG
1201 GCTCTAGAAC CAGTAGGATT AGAGAGAAAT AAGAGACACG TTCAAGAATA
1251 A
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1 MINRRYELFK DVSDADWNDW RWQVRNRIET VEELKKYIPL TKEEEEGVAQ
51 CVKSLRMAIT PYYLSLIDPN DPNDPVRKQA IPTALELNKA AADLEDPLHE
101 DTDSPVPGLT HRYPDRVLLL ITDMCSMYCR HCTRRRFAGQ SDDSMPMERI
151 DKAIDYIRNT PQVRDVLLSG GDALLVSDET LEYIIAKLRE IPHVEIVRIG
201 SRTPVVLPQR ITPELVNMLK KYHPVWLNTH FNHPNEITEE STRACQLLAD
251 AGVPLGNQSV LLRGVNDCVH VMKELVNKLV KIRVRPYYIY QCDLSLGLEH
301 FRTPVSKGIE IIEGLRGHTS GYCVPTFVVD APGGGGKTPV MPNYVISQSH
351 DKVILRNFEG VITTYSEPIN YTPGCNCDVC TGKKKVHKVG VAGLLNGEGM
401 ALEPVGLERN KRHVQE
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DNA molecules encoding the clostridial lysine 2,3-aminomutase gene can be obtained by screening cDNA or genomic libraries with polynucleotide probes having nucleotide sequences based upon SEQ ID NO:1. For example, a suitable library can be prepared by obtaining genomic DNA from *Clostridium subterminale* strain SB4 (ATCC No. 29748) and constructing a library according to standard methods. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 2-1 to 2-13 and 5-1 to 5-6 (John Wiley & Sons, Inc. 1995).

Alternatively, the clostridial lysine 2,3-aminomutase gene can be obtained by synthesizing DNA molecules using mutually priming long oligonucleotides. See, for example, Ausubel *et al.*, (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, pages 8.2.8 to 8.2.13 (1990) ["Ausubel"]. Also, see Wosnick *et al., Gene 60*:115 (1987); and Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 8-8 to 8-9 (John Wiley & Sons, Inc.

1995). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least 2 kilobases in length. Adang *et al., Plant Molec. Biol. 21*:1131 (1993); Bambot *et al., PCR Methods and Applications* 2:266 (1993); Dillon *et al.*, "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in METHODS IN MOLECULAR BIOLOGY, Vol. 15: PCR PROTOCOLS: CURRENT METHODS AND APPLICATIONS, White (ed.), pages 263-268, (Humana Press, Inc. 1993); Holowachuk *et al., PCR Methods Appl. 4*:299 (1995).

Variants of clostridial lysine 2,3-aminomutase can be produced that contain conservative amino acid changes, compared with the parent enzyme. That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NO:2, in which an alkyl amino acid is substituted for an alkyl amino acid in the clostridial lysine 2,3-aminomutase amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in the clostridial lysine 2,3-aminomutase amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in the clostridial lysine 2,3-aminomutase amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in the clostridial lysine 2,3-aminomutase amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in the clostridial lysine 2,3-aminomutase amino acid sequence, a basic amino acid is substituted for a basic amino acid in the clostridial lysine 2,3-aminomutase amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in the clostridial lysine 2,3-aminomutase amino acid sequence.

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) cysteine and methionine, (4) serine and threonine, (5) aspartate and glutamate, (6) glutamine and asparagine, and (7) lysine, arginine and histidine.

Conservative amino acid changes in the clostridial lysine 2,3-aminomutase can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO:1. Such "conservative amino acid" variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis,

mutagenesis using the polymerase chain reaction, and the like. Ausubel *et al., supra*, at pages 8.0.3-8.5.9; Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 8-10 to 8-22 (John Wiley & Sons, Inc. 1995). Also see generally, McPherson (ed.), DIRECTED MUTAGENESIS: A PRACTICAL APPROACH, IRL Press (1991). The ability of such variants to convert L-lysine to L-β-lysine can be determined using a standard enzyme activity assay, such as the assay described herein.

In addition, routine deletion analyses of DNA molecules can be performed to obtain "functional fragments" of the clostridial lysine 2,3-aminomutase. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NO:1 can be digested with *Bal*31 nuclease to obtain a series of nested deletions. The fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptides are isolated and tested for lysine 2,3-aminomutase enzyme activity. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired fragment. Alternatively, particular fragments of the clostridial lysine 2,3-aminomutase gene can be synthesized using the polymerase chain reaction. Standard techniques for functional analysis of proteins are described by, for example, Treuter *et al., Molec. Gen. Genet. 240*:113 (1993); Content *et al.*, "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in BIOLOGICAL INTERFERON SYSTEMS, PROCEEDINGS OF ISIR-TNO MEETING ON INTERFERON SYSTEMS, Cantell (ed.), pages 65-72 (Nijhoff 1987); Herschman, "The EGF Receptor," in CONTROL OF ANIMAL CELL PROLIFERATION, Vol. 1, Boynton *et al.*, (eds.) pages 169-199 (Academic Press 1985); Coumailleau *et al., J. Biol. Chem. 270*:29270 (1995); Fukunaga *et al., J. Biol. Chem. 270*:25291 (1995); Yamaguchi *et al., Biochem. Pharmacol. 50*:1295 (1995); and Meisel *et al., Plant Molec. Biol. 30*:1 (1996).

The present invention also contemplates functional fragments of clostridial lysine 2,3-aminomutases that have conservative amino acid changes.

3. Expression of Cloned Lysine 2,3-Aminomutase

To express the polypeptide encoded by a lysine 2,3-aminomutase gene, the DNA sequence encoding the enzyme must be operably linked to regulatory

sequences that control transcriptional expression in an expression vector and then, introduced into either a prokaryotic or eukaryotic host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Suitable promoters for expression in a prokaryotic host can be repressible, constitutive, or inducible. Suitable promoters are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lacλpr*, *phoA*, *gal*, *trc* and *lacZ* promoters of *E. coli,* the α-amylase and the σ^{28}-specific promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol. 1*:277 (1987); Watson *et al.*, MOLECULAR BIOLOGY OF THE GENE, 4th Ed., Benjamin Cummins (1987); Ausubel *et al., supra*, and Sambrook *et al., supra*.

Preferred prokaryotic hosts include *E. coli*, *Clostridium*, and *Haemophilus*. Suitable strains of *E. coli* include DH1, DH4α, DH5, DH5α, DH5αF', DH5αMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, BL21(DE3), BL21(DE3)plysS, BLR(DE3), BLR(DE3)plysS, and ER1647 (see, for example, Brown (Ed.), MOLECULAR BIOLOGY LABFAX, Academic Press (1991)). Suitable *Clostridia* include *Clostridium subterminale* SB4 (ATCC No. 29748) and *Clostridium acetobutylicum* (ATCC No. 824), while a suitable *Haemophilus* host is *Haemophilus influenza* (ATCC No. 33391).

An alternative host is *Bacillus subtilus*, including such strains as BR151, YB886, MI119, MI120, and B170. See, for example, Hardy, "Bacillus Cloning Methods," in DNA CLONING: A PRACTICAL APPROACH, Glover (Ed.), IRL Press (1985).

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art. See, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal

antibodies," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 15-58 (Oxford University Press 1995). Also see, Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, pages 137-185 (Wiley-Liss, Inc. 1995); and Georgiou, "Expression of Proteins in Bacteria," in PROTEIN ENGINEERING: PRINCIPLES AND PRACTICE, Cleland *et al.* (eds.), pages 101-127 (John Wiley & Sons, Inc. 1996).

An expression vector can be introduced into bacterial host cells using a variety of techniques including calcium chloride transformation, electroporation, and the like. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 1-1 to 1-24 (John Wiley & Sons, Inc. 1995).

To maximize recovery of functional lysine 2,3-aminomutase from recombinant hosts, transformed cells should be cultured under anaerobic conditions. Methods for culturing recombinant clostridia are well-known to those of skill in the art. See, for example, Mermelstein *et al., Ann. N.Y. Acad. Sci. 721*:54 (1994); Walter *et al., Ann. N.Y. Acad. Sci. 721*:69. (1994). Additionally, anaerobic culturing of bacteria is well known in the art. See, for example, Smith and Neidhardt, *J. Bacteriol.* 154:336 (1983).

4. Isolation of Cloned Lysine 2,3-Aminomutase and Production of Anti-Lysine 2,3-Aminomutase Antibodies

(a) Isolation of Recombinant Lysine 2,3-Aminomutase

General methods for recovering protein produced by a bacterial system are provided by, for example, Grisshammer *et al.*, "Purification of over-produced proteins from *E. coli* cells," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 59-92 (Oxford University Press 1995); Georgiou, "Expression of Proteins in Bacteria," in PROTEIN ENGINEERING: PRINCIPLES AND PRACTICE, Cleland *et al.* (eds.), pages 101-127 (Wiley-Liss, Inc. 1996).

Recombinant lysine 2,3-aminomutases can be purified from bacteria using standard methods that have been used to purify *Clostridium subterminale* SB4 lysine 2,3-aminomutase. In general, several precautions can be taken to ensure high enzyme activity of the purified protein. As discussed above, for example, enzyme

activity will be maximal when host cells are cultured under anaerobic conditions. Frey and Reed, *Adv. Enzymol. 66*:1 (1993). Oxygen should also be excluded during all purification steps. Purification under anaerobic conditions protects metal cofactors from being irreversibly degraded and allows maximal activity to be attained upon activation with S-adenosylmethionine.

Enzyme activity of isolated lysine 2,3-aminomutase can also be maximized by including cobalt in culture media and purification buffers. Suitable culture media, for example, contain 10 - 100 μM $CoCl_2$, while purification buffers may contain 5 μM $CoCl_2$. Culture media may also contain 10 - 100 μM Fe^{2+}. In addition, the inclusion of pyridoxal 5'-phosphate and lysine in purification buffers will aid in the stabilization of enzyme activity. For example, purification buffers may contain 10 - 100 μM pyridoxal 5'-phosphate and 100 μM lysine.

As an illustration, recombinant bacterial host cells that over-produce lysine 2,3-aminomutase can be cultured under anaerobic conditions in medium described by Chirpich *et al., J. Biol. Chem. 245*:1778 (1970), which also contains 100 μM ferric ammonium sulfate and 100 μM cobalt chloride. Typically, cells are harvested at A_{660} values of 0.5 to 0.7.

The enzyme can be purified according to the procedure of Moss and Frey, *J. Biol. Chem. 265*:18112 (1990), as modified by Petrovich *et al., J. Biol. Chem. 226*:7656 (1991). In this procedure, all steps are performed in standard buffer, which consists of 30 mM Tris-HCl (pH 8.0), 0.1 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 0.1 mM lysine, and 4.0 ml of a saturated solution of phenylmethanesulfonylflouride (in 95% ethanol) per liter of buffer. All steps are carried out at 0 - 4° C. Centrifugations can be performed in a Sorvall RC-5 centrifuge with a GSA rotor. Sonication and streptomycin sulfate precipitation steps are performed in a glove box under nitrogen. During all other steps, a stream of nitrogen or argon is maintained over the protein at all times, and all containers are flushed with argon before use. Alternatively, all steps, from cell disruption through chromatographic separations, can be performed in a nitrogen atmosphere in a Coy anaerobic chamber.

According to this method, fifty grams of bacterial cells are thawed and washed in 100 ml of standard buffer. The washed pellet is resuspended in 65 ml of standard buffer and sonicated using a Sonifier (Ultrasonics, Model W255R) at 72% of

maximum power for a total of four minutes in one minute bursts. The solution should be cooled to 4° C between bursts. After adding an additional 10 ml of buffer, the solution is centrifuged at 13,000 rpm for 30 minutes.

The supernatant fluid, including the viscous layer above the pellet, is decanted, and 25 ml of a 14% solution of streptomycin sulfate in standard buffer is added dropwise over a period of 30 minutes. The suspension is then centrifuged at 13,000 rpm for 45 minutes.

After measuring the volume of supernatant fluid, sufficient solid ammonium sulfate is added during 10 minutes to give a solution 42% saturated in ammonium sulfate, which is then stirred for an additional 40 minutes. The suspension is centrifuged for 30 minutes at 13,000 rpm, the pellet is discarded, the volume of the liquid layer is measured, and sufficient ammonium sulfate is added to give a solution 52% saturated in ammonium sulfate. After centrifugation at 13,000 rpm for 45 minutes, the resulting pellet is resuspended in 4 - 5 ml of standard buffer (final volume: 12 - 15 ml).

The isolated protein is then applied to a 100 ml column of Phenyl Sepharose equilibrated with standard buffer that also contains 2 M ammonium sulfate. The column is eluted with a linear gradient, decreasing from 2 M to 0 M ammonium sulfate in the same buffer, using a total volume of one liter, at a flow rate of 1.5 - 2 ml per minute. Ten milliliter fractions are collected. The column is then washed with an additional 250 ml of the same buffer less ammonium sulfate. The fractions containing lysine 2,3-aminomutase are located by A_{410} measurements and activity assays. The enzyme typically elutes from the column just before the end of the gradient. Active fractions are combined and the protein is concentrated by the addition of ammonium sulfate to 75% saturation, followed by stirring for 45 minutes. After centrifugation at 9,000 rpm for 40 minutes, the pellet is frozen with liquid nitrogen and stored at -70° C.

The enzyme can be purified further by ion exchange chromatography through a 50-ml column of QAE Sepharose, followed by gel permeation through a column (2.7 x 37 cm, 210 ml) of Sephacryl S-300 superfine. Petrovich *et al., J. Biol. Chem. 226*:7656 (1991).

The above procedure can be used to obtain enzyme preparations that are typically homogenous and that migrate as a single prominent band (M_r = 47,000).

Isolated lysine 2,3-aminomutase appears to be about 90% pure, although a very few faint additional bands may appear on heavily loaded SDS-PAGE gels.

Additional variations in purification are described by Petrovich *et al., J. Biol. Chem. 226*:7656 (1991), and can be devised by those of skill in the art. For example, anti-lysine 2,3-aminomutase antibodies, obtained as described below, can be used to isolate large quantities of lysine 2,3-aminomutase by immunoaffinity purification.

Lysine 2,3-aminomutase activity can be determined by measuring the conversion of radiolabeled L-lysine to radiolabeled L-β-lysine. For example, Chirpich *et al., J. Biol. Chem. 245*:1778 (1970), describe a radioenzyme assay using ^{14}C-labeled L-lysine. Briefly, an enzyme activation solution is prepared by mixing the following components in the following order: sufficient distilled water to give a final volume of 120 µl, 5.0 µl of 1.0 M Tris-HCl (pH 8.2), 5.0 µl of 1.2 mM pyridoxal phosphate, test enzyme, 5.0 µl of 0.3 M glutathione (pH 8.3), 5.0 µl of 24 mM ferrous ammonium sulfate, and 5.0 µl of 24 mM sodium dithionite. During mixing, a flow of argon should be maintained to the bottom of tubes to protect auto-oxidizable components.

Immediately after addition of dithionite, tubes are mixed gently to avoid exposure of the solution to air. An acid-washed glass capillary (14 cm long x 0.8 mm inner diameter) is filled with the activation solution until about one centimeter of free space remains at each end. After sealing both ends with a gas-oxygen torch, capillary tubes are incubated in a 37° C water bath for 60 minutes. After incubation, capillary tubes are broken at one end, and a 5 µl aliquot of activated enzyme solution is removed from the center using a 10 µl Hamilton syringe and assayed.

Components for the assay solution are added to tubes in the following order: 35 µl of distilled water, 5 µl of 0.3 M Tris-HCl (pH 7.8), 5.0 µl of 0.12 M ^{14}C-labeled L-lysine (0.033 µCi per µmole, uniformly labeled), 5.0 µl of 46 µM *S*-adenosylmethionine (in 10 mM HCl), 5 µl of 12 mM sodium dithionite, and 5 µl of activated enzyme. Just before addition of dithionite, a flow of argon is started to avoid oxidation. Each sample is sealed in a capillary tube and incubated for 15 minutes in a 30°C water bath. The reaction is stopped by adding the reaction mixture to 30 µl of 0.4 N formic acid.

Lysine and β-lysine in the acidified reaction mixture are separated by paper ionophoresis. For each determination, 5 µl of carrier β-lysine (10 mM) and two 5 µl aliquots of the acidified reaction mixture are applied along a line near the middle of a sheet of filter paper (56 x 46 cm). After ionophoresis, the amino acids are located by dipping the dried paper in 0.01% ninhydrin in acetone. The spots are cut out and counted in a scintillation counter.

The basic assay protocol of Chirpich *et al.* can be varied. For example, the activation solution can be modified by replacing glutathione with dihydrolipoate, and ferrous ammonium sulfate can be replaced with ferric ammonium sulfate. Moss and Perry, *J. Biol. Chem. 262*:14859 (1987). In another variation, the test enzyme can be activated by incubation at 30°C for six hours. Petrovich *et al.*, *J. Biol. Chem. 266*:7656 (1991). Moreover, Ballinger *et al.*, *Biochemistry 31*:949 (1992), describe several modifications of the basic method including the use of an anaerobic chamber to perform the entire procedure. Those of skill in the art can devise further modifications of the assay protocol.

(b) Preparation of Anti-Lysine 2,3-Aminomutase Antibodies and Fragments Thereof

Antibodies to lysine 2,3-aminomutase can be obtained, for example, using the product of an expression vector as an antigen. Polyclonal antibodies to recombinant enzyme can be prepared using methods well-known to those of skill in the art. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in IMMUNOCHEMICAL PROTOCOLS (Manson, ed.), pages 1-5 (Humana Press 1992). Also see, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 15-58 (Oxford University Press 1995).

Alternatively, an anti-lysine 2,3-aminomutase antibody can be derived from a rodent monoclonal antibody (MAb). Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art. *See*, for example, Kohler *et al.*, *Nature 256*:495 (1975), and Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"]. Also see, Picksley *et al.*, "Production of monoclonal antibodies

against proteins expressed in *E. coli*," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 93-122 (Oxford University Press 1995).

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

MAbs can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3. Also, see Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992).

For particular uses, it may be desirable to prepare fragments of anti-lysine 2,3-aminomutase antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent Nos. 4,036,945 and 4,331,647 and references contained therein. Also, see Nisonoff *et al., Arch Biochem. Biophys. 89*:230 (1960); Porter, *Biochem. J. 73*:119 (1959), Edelman *et al.*, in METHODS IN ENZYMOLOGY VOL. 1, page 422 (Academic Press 1967), and Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association can be noncovalent, as described in Inbar *et al., Proc. Nat'l Acad. Sci. USA 69*:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, for example, Sandhu, *Crit. Rev. Biotech. 12*:437 (1992).

Preferably, the Fv fragments comprise V_H and V_L chains which are connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow *et al., Methods: A Companion to Methods in Enzymology* 2:97 (1991). Also see Bird *et al., Science 242*:423 (1988), Ladner *et al.*, U.S. Patent No. 4,946,778, Pack *et al., Bio/Technology 11*:1271 (1993), and Sandhu, *supra*.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick *et al., Methods: A Companion to Methods in Enzymology* 2:106 (1991); Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 166-179 (Cambridge University Press 1995); and Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.*, (eds.), pages 137-185 (Wiley-Liss, Inc. 1995).

5. Isolation of Additional Lysine 2,3-Aminomutase Genes

The nucleotide sequences of the clostridial lysine 2,3-aminomutase gene and antibodies to the enzyme provide a means to isolate additional lysine 2,3-aminomutase genes. Such genes can encode enzymes from various organisms, including *Porphyromonas, Bacillus, Deinococcus, Aquifex, Treponema, Haemophilus, Escherichia*, and *Streptomyces*.

For example, the amino acid sequence of the clostridial lysine 2,3-aminomutase was used to identify related enzymes in various bacteria. Sequence analyses revealed a sequence identity of about 72%, 64%, 54%, 48%, 39%, 33% and 31% between the amino acid sequence of the clostridial enzyme and unknown gene products of *Porphyromonas gingivalis* (incomplete genome, The Institute for Genomic Research "TIGR" hypothetical protein), *Bacillus subtilus* (AF015775), *Deinococcus radiodurans* (incomplete genome, TIGR hypothetical protein), *Aquifex aeolicus* (AE000690), *Treponema pallidum* (AE001197), *Haemophilus influenza* (P44641), and *Escherichia coli* (P39280) respectively. The nucleotide and amino acid sequences (SEQ ID NOs:3 and 4) of the *E. coli* polypeptide are:

```
   1 ATGGCGCATATTGTAACCCTAAATACCCCATCCAGAGAAGATTGGTTAACGCAACTTGCC
  61 GATGTTGTGACCGATCCTGATGAACTTCTGCGTCTTTTGAATATAGACGCGGAGGAAAAA
 121 CTGTTAGCCGGACGCAGCGCCAAAAAGCTTTTTGCCCTGCGTGTGCCCCGCTCATTTATC
 181 GATCGCATGGAGAAAGGCAATCCGGACGATCCTCTTTTGCGTCAGGTACTTACCTCGCAA
 241 GATGAGTTTGTCATCGCGCCCGGATTCTCCACCGACCCACTGGAAGAACAGCACAGCGTA
 301 GTGCCTGGTTTGTTGCATAAATACCACAACCGGGCGCTTTTGCTGGTCAAAGGCGGCTGC
 361 GCGGTAAATTGCCGCTATTGCTTCCGTCGTCACTTCCCCTATGCCGAAAATCAGGGCAAC
 421 AAGCGTAACTGGCAAACTGCACTTGAGTATGTTGCTGCGCATCCGGAACTGGACGAGATG
 481 ATTTTCTCCGGCGGCGATCCGCTGATGGCGAAAGATCACGAGCTGGACTGGTTGCTCACA
 541 CAACTGGAAGCCATCCCGCATATAAAACGTCTGCGGATTCACAGCCGTCTGCCGATTGTG
 601 ATCCCGGCACGTATCACCGAGGCGCTGGTTGAATGCTTTGCCCGTTCTACGCTGCAAATC
 661 TTGCTGGTGAATCACATCAACCATGCCAATGAGGTAGATGAAACATTCCGTCAGGCGATG
 721 GCTAAGTTGCGCCGGGTAGGCGTTACTTTGCTGAACCAGAGCGTTCTGTTACGTGATGTG
 781 AACGATAACGCACAAACGCTGGCAAACCTGAGTAATGCGTTGTTCGATGCCGGCGTAATG
 841 CCGTATTACCTGCATGTGCTCGATAAAGTACAGGGCGCGGCGCATTTTATGGTGAGTGAT
 901 GACGAAGCACGGCAGATTATGCGTGAGTTGCTGACACTGGTGTCGGGATATCTGGTGCCG
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961 AAACTGGCGCGAGAAATTGGCGGCGAACCCAGCAAAACGCCGCTGGATCTCCAGCTACGC

1021 CAGCAGTAA

1 MAHIVTLNTPSREDWLTQLADVVTDPDELLRLLNIDAEEKLLAGRSAKKL

51 FALRVPRSFIDRMEKGNPDDPLLRQVLTSQDEFVIAPGFSTDPLEEQHSV

101 VPGLLHKYHNRALLLVKGGCAVNCRYCFRRHFPYAENQGNKRNWQTALEY

151 VAAHPELDEMIFSGGDPLMAKDHELDWLLTQLEAIPHIKRLRIHSRLPIV

201 IPARITEALVECFARSTLQILLVNHINHANEVDETFRQAMAKLRRVGVTL

251 LNQSVLLRDVNDNAQTLANLSNALFDAGVMPYYLHVLDKVQGAAHFMVSD

301 DEARQIMRELLTLVSGYLVPKLAREIGGEPSKTPLDLQLRQQ

The nucleotide and amino acid sequences (SEQ ID NOs: 5 and 6) of the *H. influenza* polypeptide are:

1 ATGCGTATTTTACCCCAAGAACCCGTCATTAGAGAAGAACAAAATTGGCTCACAATTCTA

61 AAAAATGCCATTTCAGATCCTAAATTATTACTAAAAGCCTTAAATTTACCAGAAGATGAT

121 TTTGAGCAATCCATTGCTGCGCGGAAACTTTTTTCGCTCCGCGTGCCACAACCTTTCATT

181 GATAAAATAGAAAAAGGTAATCCGCAAGATCCCCTTTTCTTGCAAGTGATGTGTTCTGAT

241 TTAGAGTTTGTGCAAGCGGAGGGATTTAGTACGGATCCCTTAGAAGAAAAAAATGCCAAT

301 GCGGTGCCAAATATTCTTCATAAATATAGAAATCGCTTGCTCTTTATGGCAAAAGGCGGT

361 TGTGCGGTGAATTGTCGTTATTGCTTTCGCCGACATTTTCCTTACGATGAAAACCCAGGA

421 AATAAAAAAAAGCTGGCAACTGGCGTTAGATTACATTGCGGCACATTCTGAAATAGAAGAA

481 GTGATTTTTTCAGGTGGCGATCCTTTAATGGCGAAAGATCACGAATTAGCGTGGTTAATA

541 AAACATTTGGAAAATATACCGCACTTACAACGTTTGCGTATTCACACCCGTTTGCCTGTT

601 GTGATTCCGCAACGGATTACTGATGAATTTTGCACTTTATTAGCAGAAACTCGTTTGCAA

661 ACAGTTATGGTGACACACATTAATCACCCGAATGAAATTGATCAAATTTTTGCTCATGCG

721 ATGCAAAAATTAAACGCCGTGAATGTCACGCTTTTGAATCAATCTGTTTTGCTAAAAGGC

781 GTGAATGATGATGCGCAAATTCTAAAAATATTGAGCGATAAACTTTTTCAAACAGGCATT

841 TTGCCTTATTACTTGCATTTGCTGGATAAAGTTCAAGGGGCGAGCCATTTTTTGATTAGC

901 GATATTGAAGCTATGCAAATCTATAAAACCTTGCAATCTCTGACTTCTGGCTATCTTGTT

961 CCTAAACTTGCACGAGAAATTGCGGGCGAGCCAAATAAGACTTTATACGCAGAATAA

1 MRILPQEPVIREEQNWLTILKNAISDPKLLLKALNLPEDDFEQSIAARKL

51 FSLRVPQPFIDKIEKGNPQDPLFLQVMCSDLEFVQAEGFSTDPLEEKNAN

101 AVPNILHKYRNRLLFMAKGGCAVNCRYCFRRHFPYDENPGNKKSWQLALD
151 YIAAHSEIEEVIFSGGDPLMAKDHELAWLIKHLENIPHLQRLRIHTRLPV
201 VIPQRITDEFCTLLAETRLQTVMVTHINHPNEIDQIFAHAMQKLNAVNVT
251 LLNQSVLLKGVNDDAQILKILSDKLFQTGILPYYLHLLDKVQGASHFLIS
301 DIEAMQIYKTLQSLTSGYLVPKLAREIAGEPNKTLYAE

The nucleotide and amino acid sequences (SEQ ID NOs: 7 and 8) of the *P. gingivalis* polypeptide are:

1 ATGGCAGAAA GTCGTAGAAA GTATTATTTC CCTGATGTCA CCGATGAGCA
51 ATGGAACGAC TGGCATTGGC AGGTCCTCAA TCGAATTGAG ACGCTCGACC
101 AGCTGAAAAA GTACGTTACA CTCACCGCTG AAGAAGAAGA GGGAGTAAAA
151 GAATCGCTCA AAGTACTCCG AATGGCTATC ACACCTTATT ATTTGAGTTT
201 GATAGACCCC GAGAATCCTA ATTGTCCGAT TCGTAAACAA GCCATTCCTA
251 CTCATCAGGA ACTGGTACGT GCTCCTGAAG ATCAGGTAGA CCCACTTAGT
301 GAAGATGAAG ATTCGCCCGT ACCCGGACTG ACTCATCGTT ATCCGGATCG
351 TGTATTGTTC CTTATCACGG ACAAATGTTC GATGTACTGT CGTCATTGTA
401 CTCGCCGTCG CTTCGCAGGA CAGAAAGATG CTTCTTCTCC TTCTGAGCGC
451 ATCGATCGAT GCATTGACTA TATAGCCAAT ACACCGACAG TCCGCGATGT
501 TTTGCTATCG GGAGGCGATG CCCTCCTTGT CAGCGACGAA CGCTTGGAAT
551 ACATATTGAA GCGTCTGCGC GAAATACCTC ATGTGGAGAT TGTTCGTATA
601 GGAAGCCGTA CGCCGGTAGT CCTTCCTCAG CGTATAACGC CTCAATTGGT
651 GGATATGCTC AAAAAATATC ATCCGGTGTG GCTGAACACT CACTTCAACC
701 ACCCGAATGA AGTTACCGAA GAAGCAGTAG AGGCTTGTGA AAGAATGGCC
751 AATGCCGGTA TTCCGTTGGG TAACCAAACG GTTTTATTGC GTGGAATCAA
801 TGATTGTACA CATGTGATGA AGAGATTGGT ACATTTGCTG GTAAAGATGC
851 GTGTGCGTCC TTACTATATA TATGTATGCG ATCTTTCGCT TGGAATAGGT
901 CATTTCCGCA CGCCGGTATC TAAAGGAATC GAAATTATCG AAAATTTGCG
951 CGGACACACC TCGGGCTATG CTGTTCCTAC CTTTGTGGTA GATGCTCCGG
1001 GGGGTGGTGG TAAGATACCT GTAATGCCGA ACTATGTTGT ATCTCAGTCC
1051 CCACGACATG TGGTTCTTCG CAATTATGAA GGTGTTATCA CAACCTATAC
1101 GGAGCCGGAG AATTATCATG AGGAGTGTGA TTGTGAGGAC TGTCGAGCCG
1151 GTAAGCATAA AGAGGGTGTA GCTGCACTTT CCGGAGGTCA GCAGTTGGCT

1201 ATCGAGCCTT CCGACTTAGC TCGCAAAAAA CGCAAGTTTG ATAAGAACTG

1251 A

1 MAESRRKYYF PDVTDEQWND WHWQVLNRIE TLDQLKKYVT LTAEEEEGVK

51 ESLKVLRMAI TPYYLSLIDP ENPNCPIRKQ AIPTHQELVR APEDQVDPLS

101 EDEDSPVPGL THRYPDRVLF LITDKCSMYC RHCTRRRFAG QKDASSPSER

151 IDRCIDYIAN TPTVRDVLLS GGDALLVSDE RLEYILKRLR EIPHVEIVRI

201 GSRTPVVLPQ RITPQLVDML KKYHPVWLNT HFNHPNEVTE EAVEACERMA

251 NAGIPLGNQT VLLRGINDCT HVMKRLVHLL VKMRVRPYYI YVCDLSLGIG

301 HFRTPVSKGI EIIENLRGHT SGYAVPTFVV DAPGGGGKIP VMPNYVVSQS

351 PRHVVLRNYE GVITTYTEPE NYHEECDCED CRAGKHKEGV AALSGGQQLA

401 IEPSDLARKK RKFDKN

The nucleotide and amino acid sequences (SEQ ID NOs: 9 and 10) of the *B. subtilus* polypeptide are:

1 TTGAAAAACA AATGGTATAA ACCGAAACGG CATTGGAAGG AGATCGAGTT

51 ATGGAAGGAC GTTCCGGAAG AGAAATGGAA CGATTGGCTT TGGCAGCTGA

101 CACACACTGT AAGAACGTTA GATGATTTAA AGAAAGTCAT TAATCTGACC

151 GAGGATGAAG AGGAAGGCGT CAGAATTTCT ACCAAAACGA TCCCCTTAAA

201 TATTACACCT TACTATGCTT CTTTAATGGA CCCCGACAAT CCGAGATGCC

251 CGGTACGCAT GCAGTCTGTG CCGCTTTCTG AAGAAATGCA CAAAACAAAA

301 TACGATCTGG AAGACCCGCT TCATGAGGAT GAAGATTCAC CGGTACCCGG

351 TCTGACACAC CGCTATCCCG ACCGTGTGCT GTTTCTTGTC ACGAATCAAT

401 GTTCCATGTA CTGCCGCTAC TGCACAAGAA GGCGCTTTTC CGGACAAATC

451 GGAATGGGCG TCCCCAAAAA ACAGCTTGAT GCTGCAATTG CTTATATCCG

501 GGAAACACCC GAAATCCGCG ATTGTTTAAT TTCAGGCGGT GATGGGCTGC

551 TCATCAACGA CCAAATTTTA GAATATATTT TAAAAGAGCT GCGCAGCATT

601 CCGCATCTGG AAGTCATCAG AATCGGAACA AGAGCTCCCG TCGTCTTTCC

651 GCAGCGCATT ACCGATCATC TGTGCGAGAT ATTGAAAAAA TATCATCCGG

701 TCTGGCTGAA CACCCATTTT AACACAAGCA TCGAAATGAC AGAAGAATCC

751 GTTGAGGCAT GTGAAAAGCT GGTGAACGCG GGAGTGCCGG TCGGAAATCA

801 GGCTGTCGTA TTAGCAGGTA TTAATGATTC GGTTCCAATT ATGAAAAAGC

851 TCATGCATGA CTTGGTAAAA ATCAGAGTCC GTCCTTATTA TATTTACCAA

901 TGTGATCTGT CAGAAGGAAT AGGGCATTTC AGAGCTCCTG TTTCCAAAGG

951 TTTGGAGATC ATTGAAGGGC TGAGAGGTCA TACCTCAGGC TATGCGGTTC

1001 CTACCTTTGT CGTTGACGCA CCAGGCGGAG GAGGTAAAAT CGCCCTGCAG

1051 CCAAACTATG TCCTGTCACA AAGTCCTGAC AAAGTGATCT TAAGAAATTT

1101 TGAAGGTGTG ATTACGTCAT ATCCGGAACC AGAGAATTAT ATCCCCAATC

1151 AGGCAGACGC CTATTTTGAG TCCGTTTTCC CTGAAACCGC TGACAAAAAG

1201 GAGCCGATCG GGCTGAGTGC CATTTTTGCT GACAAAGAAG TTTCGTTTAC

1251 ACCTGAAAAT GTAGACAGAA TCAAAAGGAG AGAGGCATAC ATCGCAAATC

1301 CGGAGCATGA AACATTAAAA GATCGGCGTG AGAAAAGAGA TCAGCTCAAA

1351 GAAAAGAAAT TTTTGGCGCA GCAGAAAAAA CAGAAAGAGA CTGAATGCGG

1401 AGGGGATTCT TCATGA

1 LKNKWYKPKR HWKEIELWKD VPEEKWNDWL WQLTHTVRTL DDLKKVINLT

51 EDEEEGVRIS TKTIPLNITP YYASLMDPDN PRCPVRMQSV PLSEEMHKTK

101 YDLEDPLHED EDSPVPGLTH RYPDRVLFLV TNQCSMYCRY CTRRRFSGQI

151 GMGVPKKQLD AAIAYIRETP EIRDCLISGG DGLLINDQIL EYILKELRSI

201 PHLEVIRIGT RAPVVFPQRI TDHLCEILKK YHPVWLNTHF NTSIEMTEES

251 VEACEKLVNA GVPVGNQAVV LAGINDSVPI MKKLMHDLVK IRVRPYYIYQ

301 CDLSEGIGHF RAPVSKGLEI IEGLRGHTSG YAVPTFVVDA PGGGGKIALQ

351 PNYVLSQSPD KVILRNFEGV ITSYPEPENY IPNQADAYFE SVFPETADKK

401 EPIGLSAIFA DKEVSFTPEN VDRIKRREAY IANPEHETLK DRREKRDQLK

451 EKKFLAQQKK QKETECGGDS S

The nucleotide and amino acid sequences (SEQ ID NOs: 11 and 12) of the *D. radiodurans* polypeptide are:

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   1 TGGCAAGGCG TACCCGACGA GCAGTGGTAC GACTGGAAAT GGCAGCTCAA
  51 GAACCGCATC AACAGTGTGG AGGAGTTGCA GGAAGTCCTG ACCCTCACCG
 101 AGTCCGAGTA CCGGGGTGCG TCCGCCGAGG GCATTTTCCG CCTCGACATC
 151 ACGCCGTATT TCGCGTCCCT CATGGACCCC GAAGACCCCA CCTGCCCGGT
 201 GCGCCGTCAG GTGATTCCCA CCGAGGAGGA GCTCCAGCCG TTCACCTCCA
 251 TGATGGAAGA CTCTCTCGCG GAGGATAAGC ACTCGCCCGT GCCGGGGCTG
 301 GTGCACCGCT ACCCCGACCG CGTGCTGATG CTGGTCACGA CCCAGTGCGC
 351 GAGCTACTGC CGCTACTGCA CCCGAAGCCG CATCGTGGGC GACCCCACCG
 401 AGACGTTCAA TCCCGCCGAG TATGAGGCGC AGCTCAACTA CCTGCGCAAC
 451 ACCCCGCAGG TGCGCGACGT GCTGCTTTCC GGCGGCGACC CGCTCACACT
 501 CGCGCCGAAG GTGCTGGGGC GCCTGCTTTC CGAACTTCGT AAAATCGAGC
 551 ACATCGAAAT CATCCGCATC GGCACCCGCG TGCCCGTGTT CATGCCCATG
 601 CGCGTGACCC AGGAACTGTG CGACACGCTC GCCGAACACC ATCCGCTGTG
 651 GATGAACATT CACGTCAACC ACCCCAAGGA AATCACCCCC GAAGTGGCCG
 701 AGGCGTGTGA CCGTCTGACC CGCGCGGGCG TGCCGCTCGG CAACCAGAGC
 751 GTGCTGCTGC GCGGCGTGAA CGACCACCCG GTCATCATGC AAAAGCTGCT
 801 GCGCGAGCTC GTCAAAATTC GGGTGCGCCC CTACTACATC TACCAGTGCG
 851 ACCTCGTGCA CGGCGCTGGG CACCTGCGCA CCACGGTCAG TAAGGGTCTG
 901 GAAATCATGG AATCGCTGCG CGGCCACACC TCCGGCTACA GCGTGCCGAC
 951 CTACGTGGTG GACGCGCCCG GCGGCGGCGG CAAGATTCCG GTGGCGCCCA
1001 ACTACGTGCT CTCGCACAGC CCTGAGAAGC TGATTCTGCG CAACTTCGAG
1051 GGCTACATCG CCGCCTACTC GGAGCCCACC GATTACACCG GCCCCGACAT
1101 GGCGATTCCT GACGACTGGA TTCGCAAGGA ACCCGGCCAG ACCGGCATCT
1151 TCGGCCTGAT GGAAGGCGAG CGCATTTCCA TCGAGCCG
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   1 WQGVPDEQWY DWKWQLKNRI NSVEELQEVL TLTESEYRGA SAEGIFRLDI
  51 TPYFASLMDP EDPTCPVRRQ VIPTEEELQP FTSMMEDSLA EDKHSPVPGL
 101 VHRYPDRVLM LVTTQCASYC RYCTRSRIVG DPTETFNPAE YEAQLNYLRN
 151 TPQVRDVLLS GGDPLTLAPK VLGRLLSELR KIEHIEIIRI GTRVPVFMPM
 201 RVTQELCDTL AEHHPLWMNI HVNHPKEITP EVAEACDRLT RAGVPLGNQS
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251 VLLRGVNDHP VIMQKLLREL VKIRVRPYYI YQCDLVHGAG HLRTTVSKGL

301 EIMESLRGHT SGYSVPTYVV DAPGGGGKIP VAPNYVLSHS PEKLILRNFE

351 GYIAAYSEPT DYTGPDMAIP DDWIRKEPGQ TGIFGLMEGE RISIEP

The nucleotide and amino acid sequences (SEQ ID NOs: 13 and 14) of the *A. aeolicus* polypeptide are:

1 ATGCGTCGCT TTTTTGAGAA TGTACCGGAA AACCTCTGGA GGAGCTACGA

51 GTGGCAGATA CAAAACAGGA TAAAAACTCT TAAGGAGATA AAAAAGTACT

101 TAAAACTCCT TCCCGAGGAG GAAGAAGGAA TTAAAAGAAC TCAAGGGCTT

151 TATCCCTTTG CGATAACACC TTACTACCTC TCTTTAATAA ATCCAGAGGA

201 CCCGAAGGAT CCTATAAGAC TTCAGGCAAT CCCCCGCGTT GTAGAAGTTG

251 ATGAAAAGGT TCAGTCTGCG GGAGAACCAG ACGCTCTGAA AGAAGAAGGA

301 GATATTCCGG GTCTTACACA CAGGTATCCC GACAGGGTTC TTTTAAACGT

351 CACTACCTTT TGTGCGGTTT ACTGCAGGCA CTGTATGAGA AAGAGGATAT

401 TCTCTCAGGG TGAGAGGGCA AGGACTAAAG AGGAAATAGA CACGATGATT

451 GATTACATAA AGAGACACGA AGAGATAAGG GATGTCTTAA TTTCAGGTGG

501 TGAGCCACTT TCCCTTTCCT TGGAAAAACT TGAATACTTA CTCTCAAGGT

551 TAAGGGAAAT AAAACACGTG GAAATTATAC GCTTTGGGAC GAGGCTTCCC

601 GTTCTTGCAC CCCAGAGGTT CTTTAACGAT AAACTTCTGG ACATACTGGA

651 AAAATACTCC CCCATATGGA TAAACACTCA CTTCAACCAT CCGAATGAGA

701 TAACCGAGTA CGCGGAAGAA GCGGTGGACA GGCTCCTGAG AAGGGGCATT

751 CCCGTGAACA ACCAGACAGT CCTACTTAAA GGCGTAAACG ACGACCCTGA

801 AGTTATGCTA AAACTCTTTA GAAAACTTTT AAGGATAAAG GTAAAGCCCC

851 AGTACCTCTT TCACTGCGAC CCGATAAAGG GAGCGGTTCA CTTTAGGACT

901 ACGATAGACA AAGGACTTGA AATAATGAGA TATTTGAGGG GAAGGCTGAG

951 CGGTTTCGGG ATACCCACTT ACGCGGTGGA CCTCCCGGGA GGGAAAGGTA

1001 AGGTTCCTCT TCTTCCCAAC TACGTAAAGA AAAGGAAAGG TAATAAGTTC

1051 TGGTTTGAAA GTTTCACGGG TGAGGTCGTA GAATACGAAG TAACGGAAGT

1101 ATGGGAACCT TGA

1 MRRFFENVPE NLWRSYEWQI QNRIKTLKEI KKYLKLLPEE EEGIKRTQGL

51 YPFAITPYYL SLINPEDPKD PIRLQAIPRV VEVDEKVQSA GEPDALKEEG

101 DIPGLTHRYP DRVLLNVTTF CAVYCRHCMR KRIFSQGERA RTKEEIDTMI

151 DYIKRHEEIR DVLISGGEPL SLSLEKLEYL LSRLREIKHV EIIRFGTRLP

201 VLAPQRFFND KLLDILEKYS PIWINTHFNH PNEITEYAEE AVDRLLRRGI

251 PVNNQTVLLK GVNDDPEVML KLFRKLLRIK VKPQYLFHCD PIKGAVHFRT

301 TIDKGLEIMR YLRGRLSGFG IPTYAVDLPG GKGKVPLLPN YVKKRKGNKF

351 WFESFTGEVV EYEVTEVWEP

The nucleotide and amino acid sequences (SEQ ID NOs: 15 and 16) of the *T. pallidum* polypeptide are:

1 GTGTCTATGG CTGAGTGTAC CCGGGAACAG AGAAAGAGAC GAGGTGCAGG

51 GCGTGCTGAT GAGCATTGGC GGACGTTGAG TCCTGCCTCT TGCGCGGCAG

101 ATGCGCTGAC GGAGCATATT TCTCCAGCGT ATGCGCATTT AATTGCACAA

151 GCGCAGGGCG CGGACGCGCA GGCGCTGAAA CGTCAGGTGT GCTTTGCGCC

201 ACAGGAGCGT GTGGTGCATG CTTGCGAGTG TGCCGACCCA TTGGGTGAGG

251 ACCGGTACTG CGTGACACCC TTTTTGGTGC ATCAGTATGC GAATCGTGTG

301 TTGATGTTGG CAACAGGACG TTGCTTTTCA CACTGTCGCT ATTGTTTTCG

351 CCGCGGTTTC ATCGCCCAAC GTGCAGGGTG GATCCCCAAC GAAGAGCGCG

401 AGAAGATTAT TACGTATCTT CGTGCTACCC CTTCGGTGAA GGAAATCCTG

451 GTTTCAGGTG GTGATCCACT CACTGGTTCT TTTGCACAGG TCACATCGCT

501 TTTCCGCGCA CTGCGCAGTG TAGCGCCGGA TTTGATTATT CGTCTGTGCA

551 CTCGCGCAGT CACCTTTGCT CCGCAGGCCT TTACTCCCGA GCTGATTGCG

601 TTTCTGCAGG AGATGAAGCC GGTGTGGATA ATTCCGCATA TTAATCACCC

651 GGCAGAGCTC GGTTCTACGC AGCGCGCGGT GCTCGAGGCC TGCGTAGGCG

701 CAGGCCTCCC TGTGCAATCG CAGTCGGTAC TGTTGCGCGG GGTGAACGAT

751 TCGGTAGAGA CGCTGTGCAC ACTGTTTCAC GCGCTCACTT GTCTGGGGGT

801 TAAGCCGGGG TATCTATTTC AGTTGGATTT GGCGCCTGGA ACTGGGGATT

851 TTCGTGTGCC ACTTTCTGAC ACGCTAGCTC TGTGGCGCAC ATTGAAGGAG

901 CGCCTCTCAG GGTTGTCGCT TCCCACGCTT GCGGTGGACT TGCCAGGGGG

951 TGGAGGAAAG TTTCCGCTTG TGGCATTGGC CTTGCAGCAA GATGTCACGT

1001 GGCATCAGGA ACGCGAGGCG TTCTCCGCAC GCGGCATCGA TGGCGCGTGG

1051 TACACGTACC CGTTC

1 VSMAECTREQ RKRRGAGRAD EHWRTLSPAS CAADALTEHI SPAYAHLIAQ

51 AQGADAQALK RQVCFAPQER VVHACECADP LGEDRYCVTP FLVHQYANRV

101 LMLATGRCFS HCRYCFRRGF IAQRAGWIPN EEREKIITYL RATPSVKEIL

151 VSGGDPLTGS FAQVTSLFRA LRSVAPDLII RLCTRAVTFA PQAFTPELIA

201 FLQEMKPVWI IPHINHPAEL GSTQRAVLEA CVGAGLPVQS QSVLLRGVND

251 SVETLCTLFH ALTCLGVKPG YLFQLDLAPG TGDFRVPLSD TLALWRTLKE

301 RLSGLSLPTL AVDLPGGGGK FPLVALALQQ DVTWHQEREA FSARGIDGAW

351 YTYPF

Thus, the present invention contemplates the use of clostridial enzyme sequences to identify lysine 2,3-aminomutase from other species. The present invention further contemplates variants of such lysine 2,3-aminomutases, and the use of such enzymes to prepare β-lysine.

In one screening approach, polynucleotide molecules having nucleotide sequences disclosed herein can be used to screen genomic or cDNA libraries. Screening can be performed with clostridial lysine 2,3-aminomutase polynucleotides that are either DNA or RNA molecules, using standard techniques. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, pages 6-1 to 6-11 (John Wiley & Sons, Inc. 1995). Genomic and cDNA libraries can be prepared using well-known methods. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, pages 5-1 to 5-6 (John Wiley & Sons, Inc. 1995).

Additional lysine 2,3-aminomutase genes can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the nucleotide sequences of the lysine 2,3-aminomutase genes of Clostridium, Porphyromonas, Bacillus, Deinococcus, Aquifex, Teponema, Haemophilus or Escherichia, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu *et al.*, "Use of the Polymerase Chain Reaction to Screen Phage Libraries," in METHODS IN MOLECULAR BIOLOGY, Vol. 15: PCR PROTOCOLS: CURRENT METHODS AND APPLICATIONS, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain

Reaction to Clone Gene Family Members," in METHODS IN MOLECULAR BIOLOGY, Vol. 15: PCR PROTOCOLS: CURRENT METHODS AND APPLICATIONS, White (ed.), pages 317-337 (Humana Press, Inc. 1993).

In one instance, the gene from *Bacillus subtilus* (SEQ ID NO:9) was isolated from chromosomal DNA by PCR generating an oligonucleotide insert which after the appropriate restriction digestion was cloned into the NdeI and XhoI site of pET23a(+) expression vector (Novagen, Inc., Madison, WI). This plasmid construct when placed into *E. coli* BL21 (DE3) cells (Novagen, Inc., Madison, WI) and expressed by induction with 1 mM isopropyl-beta-thiogalactopyranoside (IPTG) produced cell extracts exhibiting lysine 2,3-aminomutase activity. Cell extracts from control BL21 (DE3) cells which contained the pET23a(+) vector without the *B. subtilus* gene and cultured as above demonstrated no measurable lysine 2,3-aminomutase activity.

Anti-lysine 2,3-aminomutase antibodies can also be used to isolate DNA sequences that encode enzymes from cDNA libraries. For example, the antibodies can be used to screen λgt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 6-12 to 6-16 (John Wiley & Sons, Inc. 1995); and Margolis *et al.*, "Screening λ expression libraries with antibody and protein probes," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 1-14 (Oxford University Press 1995).

6. The Use of Lysine 2,3-Aminomutase to Produce L-β-Lysine

(a) Production of L-β-Lysine Using Purified Enzyme

Recombinant lysine 2,3-aminomutase can be purified from host cells as described above, and used to prepare enantiomerically pure L-β-lysine. An "enantiomerically pure" L-β-lysine comprises at least 87% L-β-lysine. Enantiomerically pure L-β-lysine can be prepared in batchwise reactors using soluble lysine 2,3-aminomutase. The lysine 2,3-aminomutase can then be mixed with the required cofactors: (1) ferrous sulfate or ferric ammonium sulfate; (2) pyridoxal phosphate; (3) dehydrolipoic acid, glutathione, or dithiothreitol; (4) S-

adenosylmethionine; and (5) sodium dithionite, and L-lysine at pH 8 or other appropriate pH at a temperature between 25° C to 37° C, until the production of L-lysine is at equilibrium.

Alternatively, enatiomerically pure L-β-lysine can be obtained by continuous processing using immobilized lysine 2,3-aminomutase. Lysine 2,3-aminomutase can be packed in a column and activated by the addition of cofactors and a solution containing L-lysine at pH 8 or other appropriate pH can be passed through the column at a rate that allows completion of the reaction during contact with the enzyme. The effluent from the column will contain L-β-lysine.

Both of the above methods will produce an equilibrium mixture of L-β-lysine and L-lysine in which the predominant species is L-β-lysine. The ratio of L-β-lysine to L-lysine after processing is 7:1 when performed at pH 8 at 37° C, producing enantiomerically pure L-β-lysine. Chirpich *et al.*, *J. Biol. Chem.* 245:1778 (1970). If higher purity of L-β-lysine is desired, the L-lysine can be separated from the L-β-lysine by any number of means well known in the art, including high performance chromatography procedures, such as ion exchange chromatography at an appropriate pH to take advantage of the differences in acidities of the carboxylic acid groups and the α- and β-ammonium groups of L-lysine and L-β-lysine, respectively.

(b) Production of L-β-Lysine Using Recombinant Host Cells

In an alternative approach, L-β-lysine is produced by fermentation using recombinant host cells that over-express cloned lysine 2,3-aminomutase. General methods for high level production of amino acids from cultured bacteria are well-known to those of skill in the art. See, for example, Daugulis, *Curr. Opin. Biotechnol.* 5:192 (1994); Lee, *TIBTECH 14*:98 (1996).

The gene for lysine 2,3-aminomutase can be incorporated into an *E. coli* plasmid that carries necessary markers and *E. coli* regulatory elements for overexpression of genes. When codon usage for the lysine 2,3-aminomutase gene cloned from *Clostridia* is inappropriate for expression in *E. coli*, the host cells can be cotransformed with vectors that encode species of tRNA that are rare in *E. coli* but are frequently used by *Clostridia*. For example, cotransfection of the gene *dnaY*, encoding $tRNA^{ArgAGA/AGG}$, a rare species of tRNA in *E. coli*, can lead to high-level expression of heterologous genes in *E. coli*. Brinkmann *et al.*, *Gene* 85:109 (1989) and Kane, *Curr.*

Opin. Biotechnol. 6:494 (1995). Heterologous host cells expressing lysine 2,3-aminomutase can be cultured with favorable energy, carbon and nitrogen sources under conditions in which L-lysine in the medium is absorbed by the cells and converted intracellularly into L-β-lysine by lysine 2,3-aminomutase. Unused L-β-lysine will be excreted into the growth medium. L-β-lysine can then be purified from the medium by any methods well known in the art, including high performance chromatography procedures previously described.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

Example 1

Isolation of Clostridial Lysine 2,3-Aminomutase Gene

Lysine 2,3-aminomutase was purified from *Clostridia subterminale* SB4 cells (American Type Culture Collection, Rockville, MD) according to the procedure of Moss and Frey, J. Biol. Chem. 265:18112 (1990), as modified by Petrovich et al., J. Biol. Chem. 226:7656 (1991). The purified protein (200 μM - subunit concentration) was dialyzed overnight (1 vol. protein to 1000 vol. 1 mM NaCl) and lyophilized to dryness under vacuum.

The dried lysine 2,3-aminomutase was resuspended to the original volume in 6M guanidine hydrochloride + 0.25 M tris(hydroxymethyl)aminomethane (Tris-HCl) pH 8.5 + 1 mM ethylenediaminetetraacetic acid (EDTA). The protein was then reduced with dithiothreitol (DTT) (5 fold molar excess of DTT over cysteine residues) for 3 hours at 25° C under argon atmosphere and alkylated with 4-vinylpyridine (Aldrich Chemical Co., Milwaukee, WI) (20 fold molar excess over DTT) for 90 minutes at 25° C. The protein sample was dialyzed against distilled water (1 vol. protein to 1000 vol. water) overnight at 4° C, then lyophilized to dryness. The dried protein was dissolved in 0.1 N hydrochloric acid (HCl) and subjected to cyanogen bromide (Aldrich Chemical Co., Milwaukee, WI) cleavage by the addition of 100 fold molar excess of cyanogen bromide to methionine residues under argon gas

for 24 hours at 25° C. The sample was dried by Speed-Vac (Savant Instruments, Inc., Hicksville, NY) under vacuum and redissolved in 6M guanidine hydrochloride.

Cyanogen bromide treatment of proteins produces peptide bond cleavage at the C-terminus side of methionine residues. In the process, cyanogen bromide reacts with the sulfur atom of the thioether side chain of methionine to produce homoserine (Practical Protein Chemistry, Wiley, NY, (1986) pp. 83-88). Cyanogen bromide treatment of lysine 2,3-aminomutase produced 8 major polypeptides. These polypeptides were separated from each other using high pressure liquid chromatography (HPLC) and a Vydac C_4 reverse phase column (Vydac 214TP54, 5 M, 4.6 X 250 mm, The Separations Group, Hesperia, CA). The polypeptides were first separated into five main groups using a linear gradient of 0-80% acetonitrile in 0.1% trifluoroacetic acid (TFA) in water over 60 minutes at a flow rate of 1 ml/min. at room temperature. The individual fractions were collected, dried by Speed-Vac under vacuum, reinjected into the same column and eluted with a narrow linear gradient of acetonitrile in 0.1% TFA. Five individual gradients were used to separate 8 polypeptides.

The following linear gradients of acetonitrile in 0.1% trifluoroacetic acid in water at 1 ml/min were used: peptide 1 - (5-20% 1 hr.); peptide 2 - (5-25% 1 hr.); peptide 3a - (30-42% 6 hr.); peptide 3b - (30-42% 6 hr.); peptide 4a - (33-50% 6 hr.); peptide 4b - (33-42% 6 hr.); peptide 4c - (33-42% 6 hr.); peptide 5 (45-55% 6 hr.). All peptides except peptide 3a were represented as single peaks on the chromatogram when detected at 210 nm. Peptide 3a represented approximately five unresolved peaks on the chromatogram even when the narrow elution gradient was applied. Subsequent analysis of peptide 3a by electrospray mass spectrometry (UW Biotechnology Department, Madison, WI) indicated only one peptide species of molecular weight of 6664 Da. Thus the multiple peaks observed by HPLC were the result of chromatographic artifact.

Each polypeptide fraction was analyzed for homoserine by acid (HCl) hydrolysis of the peptide, derivatization of the amino acids produced by reaction with phenylisothiocyanate, and separation and quantification of individual amino acids. Samples collected from HPLC were dried by Speed-Vac. Each peptide was dissolved in 6N HCl, placed in a vacuum hydrolysis tube (1 ml, 8 X 60 mm, Pierce Chemical, Rockford, IL), placed under vacuum, and incubated at 110° C for 24 hours.

Following hydrolysis, the samples were dried by Speed-Vac. Derivatization, separation, and quantification of amino acids were conducted according to Heinrikson et al., Anal. Biochem. 136:65 (1984). One peptide fraction containing a low level of homoserine (peptide 3a) was tentatively identified as the C-terminus peptide.

The complete protein and peptide 3a were each sequenced 12-16 amino acids downstream from the N-terminus (Michigan State University, Department of Biochemistry, Macromolecular Facility, East Lansing, MI). The amino acid sequence information was used to design degenerate oligonucleotides at the N-terminus region of the whole protein and the N-terminus region of peptide 3a which served as primers for polymerase chain reaction (PCR). The N-terminus amino acid sequence of the complete protein used for primer design was: (SEQ ID NO:17) KDVSDA corresponding to the (+) DNA strand (SEQ ID NO:18) 5'-AARGAYGTIWSIGAYGC-3' where I=INOSINE, S=G+C, W=A+T, Y=C+T, D=G+A+T, R=A+G. The N-terminus amino acid sequence of peptide 3a used for primer design was: (SEQ ID NO:19) QSHDKV corresponding to the opposite (-) strand (SEQ ID NO:20) 5'-ATIACYTTRTCRTGISWYTG-3' where I=INOSINE, Y=C+T, R=A+G, S=G+C, W=A+T.

PCR was subsequently used to generate an oligonucleotide of 1029 bases which when cloned and sequenced represented approximately 82 per cent of the entire gene of 1251 bases for lysine 2,3-aminomutase. PCR was conducted in the following manner. Chromosomal DNA from *Clostridium subterminale SB4* was prepared and purified utilizing a commercially available kit: Qiagen Genomic Tip 500/G #13343 (Qiagen, Inc., Santa Clarita, CA). After ethanol precipitation, the genomic DNA was resuspended in TE (pH 8.0) buffer (10 mM Tris-HCl pH 8.0 + 1 mM EDTA). The PCR reaction mixture (100 µl total volume) contained: *Clostridium subterminale SB4* chromosomal DNA - 2 µg; low salt PCR buffer (Stratagene, La Jolla, CA); dNTPs - 0.2 mM; oligonucleotide primers - 10 µM each; Taq Plus Long DNA Polymerase (Stratagene) - 5 units. All samples were overlayered with 100 µl mineral oil and subjected to 35 cycles of 1 min. at 94° C, 30 sec. at 37° C, 15 sec. at 50° C, and 3 min. at 72° C. After thermocycling, DNA formed during the PCR process was purified by agarose electrophoresis (2% agarose, Promega Corp., Madison, WI) in TAE buffer (0.04 M Tris-acetate pH 8.0 + 1 mM EDTA).

Following identification and excision of appropriately sized (1 kbase) ethidium bromide stained band, DNA was extracted from the agarose using Genelute Minus EtBr spin column (Supelco, Bellefonte, PA), concentrated by precipitation with ethanol and resuspended in TE pH 8.0 buffer.

DNA obtained from PCR was cloned directly into the pCR2.1 vector (TA Cloning Kit #K2000-01, Invitrogen Corp., San Diego, CA) according to manufacturer's procedure. Either 12.8 ng or 38.4 ng of PCR insert was ligated to 50 ng pCR2.1 vector overnight at 14° C. Competent *E. coli* cells (Top10F' One Shot cells - Invitrogen Corp.) were transformed with ligation mix (either 12 or 36 ng DNA per 50 µl of cells) and white colonies chosen after cells were plated on Luria broth (LB) 10 cm plates (10 gm Difco Bactotryptone, 5 gm Difco Bacto yeast extract, 10 gm NaCl, 15 gm Bactoagar per liter water; Difco Laboratories, Detroit, MI) containing carbenicillin (100 µg/ml) (Sigma Chemical Co., St. Louis, MO) and overlayered with 40 µl isopropyl-β-thiogalactopyranoside (IPTG) (100 mM) (Promega Corp., Madison, WI) and 40 µl 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) (40 mg/ml) (Promega Corp.). Selected colonies were cultured in LB (10 gm Difco Bactotryptone, 5 gm Difco Bacto yeast extract, 10 gm NaCl per liter water; Difco Laboratories) with carbenicillin (100 µg/ml) for plasmid DNA purification. Plasmid DNA was isolated by either the Qiagen Plasmid mini or midi kits (Qiagen, Inc.).

The PCR insert was sequenced in both strands beginning at the ligation sites by the radiolabeled dideoxynucleotide Sanger method (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977) using T7 Sequenase version 2.0 Sequencing Kit (Amersham Life Science, Arlington Heights, IL). The procedure produced a sequence of 1029 base pairs which represented 82 per cent of the entire gene. The remaining unknown sequence of the gene was obtained by preparing a genomic library of *Clostridium subterminale SB4* chromosomal DNA. Prior to the preparation of the genomic library, additional information was obtained regarding the composition of the peptides obtained from cyanogen bromide treatment of the reduced and alkylated lysine 2,3-aminomutase protein. The molecular weight of the intact protein and the individual peptides (both alkylated) were obtained by electrospray mass spectrometry (UW Biotechnology Dept, Madison, WI). The molecular weights obtained were: peptide 1 - 2352; peptide 2 - 1875; peptide 3a - 6664; peptide 3b - 6229; peptide 4a -

7768; peptide 4b - 7403; peptide 4c - 6972; peptide 5 - 8003. Summation of these molecular weights plus the molecular weights of two small peptides not observed by HPLC but seen from the translated base sequence (MW = 216 and 415) and the N-terminus methionine (MW = 149) plus the additional mass of replacement of 9 homoserines with 9 methionines (ΔMW = 270) and minus ten water molecules (ΔMW=180) gives a calculated molecular weight of 48,136. Within experimental error, the summation of the molecular weights of individual peptides compares with the molecular weight of the reduced and alkylated lysine 2,3-aminomutase protein of 48, 281 obtained by electrospray mass spectrometry.

Comparison of the molecular weights of the peptides from mass spectrometry with the molecular weights of the peptides produced by translation of the known incomplete base sequence (1029 base pairs) of the protein identified all but two of the peptides. These peptides were peptide 3a and peptide 2. Since the N-terminus sequence of peptide 3a had been used for PCR to produce the sequence of 1029 base pairs and all other peptides except peptide 2 had been identified in this known sequence, peptide 2 must be the C-terminus peptide. Both peptides 2 and 3a were subjected to extensive N-terminus amino acid sequence analysis (Michigan State University, Department of Biochemistry, Macromolecular Facility, East Lansing, MI). Furthermore, C-terminus amino acid sequence analysis was conducted on the whole protein. For peptide 3a, the N-terminal amino acid sequence reported was: (SEQ ID NO:21) PNYVISQSHDKVILRNFEGVITTYSEPINYTPGCNCDVCTGKKKVHKV. For peptide 2, the N-terminal amino acid sequence reported was: (SEQ ID NO:22) ALEPVGLERNKRHVQ. For the whole protein, the N-terminus amino acid sequence reported was: (SEQ ID NO:23) MINRRYELFKDVSDAD and the C-terminus amino acid sequence reported was: EQV.

A nondegenerate, nonradioactive probe (500 bases) containing digoxygenin dUMP residues randomly incorporated was prepared by PCR (The PCR DIG PROBE Synthesis kit - #1636-090 Boehringer-Mannheim, Indianapolis, IN). The digoxygenin dUMP groups replace thymidine in some of the positions of the DNA. The following primers were used for the PCR Probe Synthesis kit: Primer 1 (+) strand (SEQ ID NO:24) - 5'-ATCCTAACGATCCTAATGATCC; Primer 2 (-) strand (SEQ ID NO:25) - 5'-TGGATGGTTAAAGTGAGTG. Using as template a plasmid containing the incomplete lysine 2,3-aminomutase gene, the following probe labeled

with digoxygenin groups was prepared: (SEQ ID NO:26) 5'-
ATCCTAACGATCCTAATGATCCAGTAAGAAAACAAGCTATTCCAACAGCATTAGAGCTTAACAAAGCT
GCTGCAGATCTTGAAGACCCATTACATGAAGATACAGATTCACCAGTACCTGGATTAACTCACAGATAT
CCAGATAGAGTATTATTATTAATAACTGATATGTGCTCAATGTACTGCAGACACTGTACAAGAAGAAGA
TTTGCAGGACAAAGCGATGACTCTATGCCAATGGAAAGAATAGATAAAGCTATAGATTATATCAGAAA
TACTCCTCAAGTTAGAGACGTATTATTATCAGGTGGAGACGCTCTTTTAGTATCTGATGAAACATTAGA
ATACATCATAGCTAAATTAAGAGAAATACCACACGTTGAAATAGTAAGAATAGGTTCAAGAACTCCAG
TTGTTCTTCCACAAAGAATAACTCCAGAACTTGTAAATATGCTTAAAAAATATCATCCAGTATGGTTAA
ACACTCAC TTTAACCATCCA-3'. Primers (1 μM) were used with plasmid template (1 ng) for PCR according to manufacturer's specifications (Boehringer-Mannheim, Indianapolis, IN). The PCR product, checked by agarose gel electrophoresis, was used directly in probe analysis.

Clostridium subterminale SB4 chromosomal DNA was isolated as described previously and subjected to restriction digestion using several restriction endonucleases. These enzymes did not cut in the region of the known lysine 2,3-aminomutase gene sequence. However, these sites were present in the multicloning region of pUC19 vector. The enzymes used were EcoRI (New England Biolabs, Beverly, MA), XbaI (Promega Corp., Madison, WI), AccI (New England Biolabs, Beverly, MA), and NdeI (Promega Corp., Madison, WI). Restriction enzyme (100 units) was reacted with chromosomal DNA (10 μg) and appropriate buffer (manufacturers specification) + 0.01% bovine serum albumin for 90 min. at 37° C in eight replicates. After restriction digestion, each fraction was applied to a preparative agarose gel (14 x 14 cm) in multiple lanes in TAE buffer (0.04 M Tris-acetate pH 8.0 + 1 mM EDTA) and subjected to electrophoresis at 150 volts. After electrophoresis, several lanes were separated from the remaining gel for probe analysis, treated with NaOH (0.5 N) solution to denature DNA, neutralized with 0.5 M Tris-HCl buffer pH 7.5, in preparation for blotting by diffusion. To the surface of this gel, nylon membrane (#1209-299 Boehringer-Mannheim, Indianapolis, IN) was applied followed by filter paper and a stack of paper towel. After 24 hr., the paper towel was removed and the nylon membrane treated for digoxygenin dUMP labeled probe analysis according to manufacturer's procedure (Boehringer Mannheim, Indianapolis, IN). Positive probe-template interaction was identified by chemiluminescence from an anti-digoxygenin antibody conjugate containing alkaline phosphatase and reacting with CDP-Star (disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)

tricyclo [3.3.1.1.]decan}-4-yl)-1-phenyl phosphate), a chemiluminescent substrate (both obtained from Boehringer-Mannheim, Indianapolis, IN). The restriction digestion produced fragments of chromosomal DNA showing positive chemiluminescent probe-template interaction of the following sizes: XbaI - 4.3 kb, EcoRI - 4.5 kb, AccI - 5.9 kb, and NdeI - 6.1 kb. From this information, the appropriate sized fragments of DNA were cut out of zones of the remaining agarose gel. DNA was extracted from these agarose bands by use of spin columns (GenElute Agarose spin column #5-6500, Supelco, Bellefonte, PA) by centrifugation at 12,000 x g for 10 min. and concentrated by ethanol precipitation.

Chromosomal DNA fragments were ligated to pUC19 plasmid vector (New England Biolabs, Beverly, MA) cut with the same restriction endonuclease and dephosphorylated, transformed into competent *E. coli* XL-2 Blue Ultracompetent cells (#200151, Stratagene, La Jolla, CA), and plated on LB agar + carbenicillin + X-Gal + IPTG (as previously described). PUC19 plasmid vector (10 μg) was incubated with respective restriction enzymes (2 units) in appropriate buffer (manufacturer's specification) + 0.01% bovine serum albumin for 1 hour at 37° C. Restriction enzyme activity was removed from the medium either by passage through a Micropure EZ Enzyme Spin column (Amicon, Inc., Beverly, MA) or by heat inactivation at 65° C for 20 min. Each restriction digested pUC19 plasmid was dephosphorylated by treatment with 1 unit of calf intestine alkaline phosphatase (Pharmacia Biotech., Piscataway, NJ) in appropriate buffer (manufacturer's specification) for 30 min. at 37° C. Alkaline phosphatase was removed by using a Micropure EZ Spin column. Plasmid DNA was purified by agarose electrophoresis in TAE buffer (as previously described). After ethidium bromide staining, appropriate size fragments of DNA (approximately 2600 base pairs) were cut out of the agarose. DNA was extracted from the agarose bands with spin columns (GenElute Minus EtBr Spin column, #5-6501, Supelco, Bellefonte, PA) by centrifugation at 14,000 x g for 20 min. and concentrated by ethanol precipitation.

For ligation, 10 ng of restriction endonuclease cut and alkaline phosphatase dephosphorylated vector was ligated to the following chromosomal DNA inserts to produce a 1:1 or 1:3 ratio of vector DNA to insert DNA: XbaI - 16 and 48 ng, EcoRI - 17 and 50 ng, AccI - 22 and 66 ng, and NdeI - 23 and 68 ng each in a total volume of 10 μl. T4 DNA ligase (3 units – Pharmacia Biotech, Piscataway, NJ)

was added to T4 DNA ligase buffer (Promega Corp., Madison, WI) and ligation occurred for 16 hours at 14° C. Transformed *E. coli* XL-2 Blue Ultracompetent cells from individual plated white colonies (approximately 500 per trial) were placed on nylon membranes, treated with alkali to expose and denature DNA, and hybridized with the oligonucleotide probe labeled with digoxygenin dUMP (procedures according to manufacturer's specifications, Boehringer-Mannheim, Indianapolis, IN). Colonies (1 or 2 per 500) in which the digoxygenin labeled probe demonstrated positive chemiluminescence when examined by X-ray film were chosen for further screening by DNA sequencing. The start codon, ATG, was found in one XbaI colony (X158). The start (ATG) and the stop (TAA) codon were found in one EcoRI colony (E138). Double stranded DNA from these selected colonies were sequenced using the automated ABI Prism Dye Terminator Cycle Sequencing procedure by the University of Wisconsin Biotechnology Department, Madison, WI to obtain the final sequence of the *Clostridium subterminale SB4* gene. The DNA sequence was translated into the amino acid sequence according to the genetic code. Amino acid sequences obtained from N-terminal and C-terminal amino acid analysis of the protein and the cyanogen bromide derived peptides were in perfect agreement with the translated DNA sequence. The molecular weight of the translated sequence of amino acids (47,025) agreed within experimental error with the molecular weight of *Clostridial* lysine 2,3-aminomutase protein obtained by electrospray mass spectrometry (47,173).

Example 2

Incorporation of Clostridia subterminale SB4 Lysine 2,3-aminomutase Gene Into E.coli.

One of the *E. coli* colonies containing the pUC19 plasmid with the nucleotide sequence encoding the entire *Clostridial* lysine 2,3-aminomutase gene from the genomic library (E138) was used to prepare an expression vector. The *Clostridial* lysine 2,3-aminomutase gene was inserted into two commercially available plasmid expression vectors. The plasmid vector, pET-23a(+) (Novagen, Inc., Madison, WI) derived from pBR322 contains the T7 promoter as well as the ribosome binding site of the phage T7 major capsid protein upstream from the multi-cloning site. The gene for *Clostridial* lysine 2,3-aminomutase was inserted into the multi-cloning site. This expression system when cloned into a cell line which produces an IPTG (isopropyl-β-

thiogalactopyranoside)-inducible T7 RNA polymerase has been reported to yield very high levels of many heterologous gene products (Studier et al., Gene Expression Technology *in* Methods in Enzymology 185:60 (1991). The plasmid vector, pKK223-3 (Amersham Pharmacia Biotech, Piscataway, NJ) also derived from pBR322 contains a strong tac promoter upstream from the multiple cloning site and a strong *rrnB* ribosomal terminator downstream. In lac I^q *E. coli* cells, the tac promoter is inducible with IPTG, although uninduced cells will show a low level of expression of the cloned gene. Both plasmids confer ampicillin resistance to *E. coli* cells.

In order to splice the lysine 2,3-aminomutase gene into the above vectors so that the start codon is correctly spaced from the respective ribosome binding site of the vector, PCR was used to generate inserts which after appropriate restriction digestion could be cloned directly into the multicloning site of each vector. The following primers for PCR were used: for pET-23a(+): (SEQ ID NO:27) (+) strand 5' - TACACATATGATAAATAGAAGATATG - 3', (SEQ ID NO:28) (-) strand 5' - TAGACTCGAGTTATTCTTGAACGTGTCTC - 3'; for pKK223-3, (SEQ ID NO:29) (+) strand 5' - TACAGAATTCATGATAAATAGAAGATATG - 3', (SEQ ID NO:30) (-) strand 5' - TAGAAAGCTTTTATTCTTGAACGTGTCTC - 3'. The DNA template used was the pUC19 plasmid with the nucleotide sequence encoding the entire *Clostridial* lysine 2,3-aminomutase gene from the genomic library (E138). pUC19 plasmid DNA was isolated by the Qiagen Plasmid mini kit (Qiagen, Inc., Santa Clarita, CA). PCR was conducted as described previously. The PCR reaction mixture (100 µl total volume) contained: pUC19 plasmid DNA - (400 ng); cloned Pfu DNA polymerase reaction buffer (Stratagene, La Jolla, CA); dNTPs - 0.2 mM each; oligonucleotide primers - 1 µM each; cloned Pfu DNA polymerase (Stratagene, La Jolla, CA) - 5 units. All samples were overlayered with 100 µl mineral oil and subjected to 35 cycles of 1 min. at 94° C, 30 sec. at 37° C, 15 sec. at 50° C, and 3 min. at 72° C. After thermocycling, DNA formed during the PCR process was further purified by agarose electrophoresis (2% agarose, Promega Corp., Madison, WI) in TAE buffer (0.04 M Tris-acetate pH 8.0 + 1 mM EDTA). Following identification and excision of the appropriately sized (~ 1.2 kbase) ethidium bromide stained band, DNA was extracted from the agarose using the GenElute Minus EtBr spin column (Supelco, Bellefonte, PA), concentrated by precipitation with ethanol, and resuspended in TE pH 8.0 buffer. The purified PCR product was blunt-end ligated to pCR-Script

Amp cloning vector (#211188 Stratagene, La Jolla, CA) using 0.3 pmoles insert to 0.005 pmoles vector according to manufacturer's specifications. The ligated DNA was used to transform XL1-Blue MRF' *E. coli* cells (Stratagene, La Jolla, CA) which were subsequently plated on LB + carbenicillin + IPTG + X-Gal plates (as previously described) and cultured overnight. White colonies were chosen and subcloned in LB + carbenicillin (100 μg/ml) media for plasmid purification.

Plasmid DNA was purified using Qiagen Plasmid mini kit (Qiagen, Inc., Santa Clarita, CA) and subjected to restriction digestion. For the pET-23a(+) insert, 10 μg of plasmid DNA was cut with NdeI (Promega Corp. Madison, WI) - 50 units and Xho I (Promega Corp.) - 50 units in a total volume of 100 μl for 1 hr. at 37° C; for pKK223-3 insert, 10 μg of plasmid DNA was cut with EcoRI (New England Biolabs, Beverly, MA) - 100 units and HindIII (New England Biolabs) - 100 units in a total volume of 100 μl for 90 min. at 37° C. The insert DNA was separated from the plasmid DNA by agarose gel electrophoresis (2% agarose in TAE buffer), purified and concentrated as previously described. The expression vectors, pET-23a(+) - 10 μg and pKK223-3 - 10 μg were similarly cut with NdeI – Xho I and EcoRI -HindIII respectively (as previously described). Additionally the restriction cut vectors were dephosphorylated at the 5' end with calf-intestine alkaline phosphatase (Promega Corp, Madison, WI) - 1 unit for 30 min. at 37° C, purified by agarose gel electrophoresis and concentrated by ethanol precipitation (as previously described). The pET-23a(+) insert and the pET-23a(+) cut vector were ligated with T4 DNA ligase (Promega Corp.). To 3 ng of insert were added 10 ng of cut vector in T4 DNA ligase buffer (Promega Corp.) + T4 DNA ligase (Promega Corp.) - 3 units in a total volume of 10 μl and incubated for 16 hr. at 14° C. The pKK223-3 insert and the pKK223-3 cut vector were ligated as previously described. Competent *E. coli* cells (Epicurian coli XL2-Blue MRF', Stratagene, La Jolla, CA) were transformed with 2 μl ligation mix and plated on LB + carbenicillin (100 μg/ml) plates. Individual colonies were subcultured in LB + carbenicillin (100 μg/ml) medium and plasmid DNA isolated using the Qiagen Plasmid DNA mini kit. The insert was sequenced in entirety including both regions of the start and stop codon by the automated ABI Prism Dye Terminator Cycle Sequencing procedure (Perkin-Elmer, Norwalk, CT) by the UW Biotech Dept (Madison, WI) to confirm the correctness of the construct.

For protein expression, the pET-23a(+) - gene insert expression vector was transformed into competent BL21(DE3) *E. coli* cells (Novagen, Madison, WI). This cell line is a λDE3 lysogen carrying the gene for T7 RNA polymerase under control of IPTG. For transformation, 20 μl of competent cells were treated with 0.1 μg of plasmid DNA. After transformation, 10 μl of cells were plated on LB + carbenicillin (100 μg/ml) + plates and grown overnight at 37° C. Individual colonies were subcultured in LB + carbenicillin (100 μg/ml) overnight at 37° C and ± 1 mM IPTG for 3 additional hours. For protein expression, the pKK223-3 - gene insert expression vector was used with the Epicurian coli XL2-Blue MRF' (Stratagene, La Jolla, CA) without transfer to another cell line or placed in *E. coli* JM109 cells. In the latter case, 100 μl of competent JM109 cells (Stratagene, La Jolla, CA) were treated with 5 ng of plasmid DNA and the cells transformed, plated, and subcultured as previously described.

Evaluation of the codon usage for the Clostridial lysine 2,3-aminomutase gene indicated that the most frequently used codon for arginine (AGA) is one of the most infrequently used codons in *E. coli*. There are 29 AGA codons for 29 total arginines with two regions containing two or three repeat AGA near the start codon. From the studies of Kane, Current Opinion in Biotech. 6:494 (1995) and Brinkmann, et al., Gene 85:109 (1989), the expression of heterologous genes containing a high frequency of rare codons (particularly AGG and AGA) in *E. coli* is difficult or impossible due to low cellular concentrations of the respective tRNA. Brinkmann *et al.* suggest that the presence of rare AGA codon usage can be relieved by overexpression of the *E. coli dnaY* gene, which supplies this minor arginine tRNA. The sequence of the *E. coli dnaY* gene was published by Garcia *et al.*, Cell 45:453 (1986). The primary products of this gene are RNAs of 180 and 190 nucleotides which are processed *in vivo* to form the mature arginine tRNA of 77 nucleotides.

Cotransfection of *E. coli* BL21 (DE3) cells with both vectors (pET23a(+) vector and pAlter-EX2 vector containing the *dnaY* gene) was not required for expression of the *Clostridial* lysine 2,3-aminomutase gene in *E. coli*. However, lysine 2,3-aminomutase activity of *E. coli* cellular extracts without pAlter-Ex2/*dnaY* were approximately 80% less than cellular extracts with this construct. The specific activity of the purified enzyme isolated from cells without pAlter-Ex2/*dnaY* was

approximately half of that of the enzyme isolated from cells containing the *dnaY* gene. The yield of purified enzyme from equivalent amounts of cells was also decreased by 65% when *dnaY* was absent. Furthermore, cell growth in the absence of the vector containing the *dnaY* gene was significantly decreased. The doubling time of cultured *E. coli* cells containing the pET 23a(+) vector during expression of the lysine 2,3-aminomutase gene was approximately four times the doubling time of the same *E. coli* cells with the additional pAlter-Ex2 vector containing the *dnaY* gene. Therefore, for long-term stability and maximal expression, *E. coli* cells containing both expression vectors were prepared. The *dnaY* gene was isolated from *E. coli* chromosomal DNA by PCR. Primers were prepared which produced a 327 bp insert containing BamHI and EcoRI restriction sites necessary for cloning into pAlter-Ex2 plasmid vector (Promega Corp.). This vector has a p15a origin of replication which allows it to be maintained with *colE*1 vectors such as pET-23a(+) and pKK223-3. Also the presence of this vector confers tetracycline resistance to *E. coli*. The PCR primers used for pAlter-Ex2 were: (SEQ ID NO:31) (+) strand - 5' – TATAGGATCCGACCGTATAATTCACGCGATTACACC - 3', (SEQ ID NO:32) -) strand - 5' - TAGAGAATTCGATTCAGTCAGGCGTCCCATTATC - 3'.

Chromosomal DNA from *E. coli* JM109 cells (Stratagene, La Jolla, CA) was prepared and purified utilizing the Qiagen Genomic Tip 500/G #13343 (Qiagen, Inc., Santa Clarita, CA). After ethanol precipitation, the genomic DNA was resuspended in TE (pH 8.0) buffer. The PCR reaction mixture (100 µl total volume) contained: *E. coli* chromosomal DNA - 2.5 µg; cloned Pfu DNA polymerase reaction buffer (Stratagene, La Jolla, CA); dNTPs - 0.2 mM each; oligonucleotide primers - 1 µM each; cloned Pfu DNA polymerase (Stratagene, La Jolla, CA) - 5 units. All samples were overlayered with 100 µl mineral oil and subjected to 35 cycles of 1 min. at 94° C, 30 sec. at 37° C, 15 sec. at 50° C, and 3 min. at 72° C. After thermocycling, DNA formed during the PCR process was further purified by agarose electrophoresis (2% agarose, Promega Corp., Madison, WI) in TAE buffer (0.04 M Tris-acetate pH 8.0 + 1 mM EDTA). Following identification and excision of the appropriately sized (~320 base pairs) ethidium bromide stained band, DNA was extracted from the agarose using the GenElute Minus EtBr spin column (Supelco, Bellefonte, PA) concentrated by precipitation with ethanol, and resuspended in TE pH 8.0 buffer.

The purified PCR product was blunt-end ligated to pCR-Script Amp cloning vector (Stratagene, La Jolla, CA) using 0.3 pmoles insert to 0.005 pmoles vector according to manufacturer's specifications. The ligated DNA was used to transform XL1-Blue MRF'·*E. coli* cells (Stratagene, La Jolla, CA) which were subsequently plated on LB + carbenicillin + IPTG + X-Gal plates (as previously described) and cultured overnight. White colonies were chosen and subcloned in LB + carbenicillin (100 μg/ml) media for plasmid purification. Plasmid DNA was purified using Qiagen Plasmid mini kit (Qiagen, Inc., Santa Clarita, CA) and subjected to restriction digestion. For the pAlter-Ex2 insert, 1 μg of plasmid DNA was cut with BamHI (Promega Corp., Madison, WI) - 10 units and EcoRI (Promega Corp.) - 10 units in a total volume of 100 μl for 1 hr. at 37° C. The insert DNA was separated from the plasmid DNA by agarose gel electrophoresis (3% agarose in TAE buffer) and purified and concentrated as previously described. The expression vector, pAlter-Ex2 - 10 μg was similarly cut with BamHI and EcoRI (as previously described). Additionally the restriction cut vector was dephosphorylated at the 5' end with calf-intestine alkaline phosphatase (Promega Corp., Madison, WI) - 10 units for 1 hr. at 37° C, purified by agarose gel electrophoresis and concentrated by ethanol precipitation (as previously described). The *dnaY* insert and the pAlter-Ex2 cut vector were ligated with T4 DNA ligase (Promega Corp.). To 1.68 ng of insert were added 10 ng of cut vector in T4 DNA ligase buffer (Promega Corp.) + T4 DNA ligase (Promega Corp.) - 3 units in a total volume of 10 μl and incubated for 16 hr. at 14° C. Competent BL21(DE3) cells (Novagen, Madison, WI) were transformed with 1 μl of ligation mix and plated on LB + tetracycline (12.5 μg/ml). Individual colonies were subcultured in LB + tetracycline (10 μg/ml) medium and plasmid DNA isolated using the Qiagen Plasmid DNA mini kit. The insert was sequenced completely by the dideoxy NTP method previously described to confirm the correctness of the construct and found to agree with the expected sequence.

BL21(DE3) cells with the pAlter-Ex2 vector (*dnaY* gene) were cotransfected with pET-23a(+) (lysine 2,3-aminomutase gene). Competent BL21(DE3) cells containing the pAlter-Ex2 *dnaY* gene insert were prepared as follows: *E. coli* cells were grown overnight in LB + tetracycline (10 μg/ml). These cells were used to innoculate a fresh culture of LB + tetracycline to give a starting absorbance at 600 nm

of 0.1. The cells were cultured at 37° C with shaking until reaching an absorbance of 0.6. Forty ml of this culture were transferred to a centrifuge tube and centrifuged: at 2000 x g for 10 min. at 4° C. To the cell pellet was added 10 ml of ice cold 0.1 M $MgCl_2$. The cell pellet was gently resuspended and incubated on ice for 20 min. followed by another centrifugation at 2000 x g for 10 min. at 4° C. To the cell pellet was added 2.5 ml of ice cold 0.1 M $CaCl_2$. The cell pellet was gently resuspended and incubated on ice for an additional 40 min.

The above competent BL21(DE3) cells containing the p-Alter-EX2 vector (*dnaY* gene) were then cotransformed separately with pET23a(+) plasmid DNA (lysine 2,3-aminomutase gene). To 20 µl of competent cells on ice was added 0.1 µg of pET23a(+) plasmid DNA. The sample was incubated on ice for 30 min. followed by a 45 sec. heat shock at 42° C and cooling on ice for 2 additional min. SOC medium (80 µl) was added and the cells incubated at 37° C with shaking at 220 rpm for 1 hr. The cells were plated on LB + carbenicillin (100 µg/ml) + tetracycline (12.5 µg/ml) and cultured overnight. Individual colonies were subcultured in LB + carbenicillin (100 µg/ml) + tetracycline (10 µg/ml) overnight at 37° C.

Example 3

Expression of Clostridia subterminale SB4 Lysine 2,3-aminomutase Gene in E. coli.

Expression of the cloned gene *Clostridial* lysine 2,3-aminomutase gene in *E. coli* was ascertained by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). A 1 ml aliquot of final cell stocks [*E. coli* BL21(DE3) cells with pET-23a(+) (lysine 2,3-aminomutase gene) ± p-Alter-EX2 vector (*dnaY* gene)] or [*E. coli* JM109 or Epicurian coli XL2-Blue MRF' with pKK223-3 (lysine 2,3-aminomutase gene)] ± IPTG was centrifuged at 14,000 x g for 10 min. at 4° C to remove cell culture media. The cell pellet was resuspended in 0.5 ml of 10 mM of 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) pH 7.5 buffer containing 0.6 mM $CaCl_2$ and 50 units deoxyribonuclease I (#D-4527, Sigma Chemical, St. Louis, MO). Following cell breakage by sonication using the micro-tip of the Sonic Dismembrator (setting 3 for three 15 sec intervals) (Model #550, Fisher Scientific, Pittsburgh. PA), 30 µl of sonicated cells were added to 100 µl of SDS PAGE sample buffer (0.06 M Tris-HCl pH 6.8 buffer containing 10%(v/v) glycerol, 0.7 M β-

mercaptoethanol, 0.025 M bromophenol blue). The cell extract was heated at 95° C for 5 min. prior to loading (5-20 μl/lane) on a mini polyacrylamide gel (Ready Gel #161-1106, Bio-Rad Laboratories, Hercules, CA), run at 150 volts (Ready Gel Cell #165-3125, Bio-Rad Laboratories, Hercules, CA) at constant voltage until the tracking dye was at the bottom of the gel, and stained with Coomassie Blue R-250 stain. Control cell extracts were prepared containing *E. coli* BL21 (DE3) cells with pET-23a(+) without the gene for lysine 2,3-aminomutase. Analysis of the stained SDS PAGE gel revealed one intensely stained band corresponding to a molecular weight of 47 kDa migrating between 40 and 50 kDa standard proteins (Benchmark Protein Ladder #10747-012, Life Technologies, Gaithersburg, MD) in all samples containing pET 23a(+) or pKK223-3 expression vectors + *Clostridial* lysine 2,3-aminomutase gene. This band migrated with the same R_f as purified *Clostridial* lysine 2,3-aminomutase. Only a weakly stained band was present in control cell extracts with the above expression vectors without the lysine 2,3-aminomutase gene.

A requirement for an anaerobic environment when measuring lysine 2,3-aminomutase activity (ie., formation of L-β-lysine from L-α-lysine) was previously demonstrated for the Clostridial enzyme [Moss and Frey, J. Biol. Chem. 265:18112 (1990), Petrovich et al., J. Biol. Chem. 226:7656 (1991)]. Therefore all subsequent steps including cell culture, cell extract preparation, and enzyme assay were done in the absence of oxygen. The following procedure demonstrates the formation of L-β-lysine from L-α-lysine *in vivo* in *E. coli* cells. BL21(DE3) cells containing the pET23a(+) expression vector for the *Clostridial* lysine 2,3-aminomutase gene with the expression vector for *E. coli dnaY* gene were cultured anaerobically at 37° C in 100 ml of M9 medium (0.68 gm Na_2HPO_4, 0.3 gm KH_2PO_4, 0.05 gm NaCl, 0.1 gm NH_4Cl) containing $CaCl_2$ (0.1 mM), $MgSO_4$ (1 mM), $ZnSO_4$ (10 μM), $Fe(II)SO_4$ (50 μM), D-(+)-glucose (0.2% w/v), ampicillin (100 μg/ml) ± tetracycline (10 μg/ml) in 150 ml sealed bottles made anaerobic by sparging with nitrogen gas and the addition of 1 mM sodium dithionite and 4 mM sodium thioglycolate (Sigma Chemical Co., St. Louis, MO). After cells reached a density of approximately 0.5 OD units at 600 nm, L-α- lysine (50 mM) was added and the cells cultured an additional 16 hrs. at 37° C anaerobically. Cells were harvested by centrifugation at 6,000 x g for 10 min. and resuspended in 0.5 ml of distilled water.

Following sonication using the micro-tip of the Sonic Dismembrator (setting 3 for three 15 sec. intervals) (Model #550- Fisher Scientific, Pittsburgh. PA), the lysed cells were centrifuged at 14,000 x g for 20 min. at room temperature. The supernatant was used to measure formation of L-β-lysine from L-α-lysine resulting from the expression of the *Clostridial* lysine 2,3-aminomutase gene in *E. coli*. Control cells which contained pET 23a(+) plasmid without the *Clostridial* lysine 2,3-aminomutase gene were also cultured and harvested as previously described.

The presence of L-β-lysine in *E. coli* cell extract was detected by treating the extract with phenylisothiocyanate (Pierce Chemical Co., Rockford, IL) which derivatizes amino acids to their respective phenylthiocarbamyl derivatives. These compounds are readily separated and detected by high pressure liquid chromatography (HPLC). The procedure is based on the method of Heinrikson and Meredith, Anal. Biochem. 136:65 (1984): 10 μl of cell extract (see above) were treated with 100 μl of coupling buffer (acetonitrile:pyridine:triethylamine:water 10:5:2:3 v/v/v) and evaporated to dryness using a Speed-Vac (Savant Instruments, Inc., Hicksville, NY). The sample was redissolved in 100 μl coupling buffer and 5 ml of phenylisothiocyanate was added and mixed. After 5 min. at room temperature, the sample was again dried using the Speed-Vac. The dried sample was redissolved in distilled water (200 μl) and centrifuged at 14,000 x g for 10 min. to remove undissolved material. The sample was injected into a Waters HPLC (Millipore Corporation, Waters Chromatography Division, Milford, MA) equipped with a Vydac C_8 reverse phase column (Vydac 208TP54, 5 mM, 4.6 x 250 mm, The Separations Group, Hesperia, CA). The derivatized L-α-lysine and L-β-lysine were separated using a linear gradient composed of buffer A (0.05 M ammonium acetate in water) and buffer B (0.1 M ammonium acetate in acetonitrile:methanol:water (46:10:44 v/v/v) at a flow rate of 1 ml/min. at room temperature and monitored at a wavelength of 254 nm. The initial conditions were 30% buffer B for 2 min. followed by a linear gradient to 60% buffer B in 24 min. The retention times for phenylthiocarbamyl derivatives of L-α-lysine was 25.7 ± 0.3 min. and for L-β-lysine was 22.9 ± 0.4 min. L-β-lysine (up to 35% of total lysine) was observed in all cell extracts of *E. coli* cells containing the pET 23a(+) plasmid vector with the *Clostridial* lysine 2,3-aminomutase gene and

absent in control cells which were treated identically but did not contain the plasmid with the *Clostridial* lysine 2,3-aminomutase gene.

In vitro formation of β-lysine by *E. coli* cell extracts was also measured utilizing the standard assay procedure (Ballinger et al., Biochemistry 31:10782 (1992). The conversion of radiolabeled C-14 L-α-lysine to radiolabeled C-14 L-β-lysine was observed in the following manner:

Aerobically grown *E. coli* cells (1 ml) containing the pET 23a(+) plasmid vector with the Clostridial lysine 2,3-aminomutase gene and the p-Alter-EX2 plasmid vector with the *E. coli dnaY* gene were used to seed a glass fermentor (Virtis Laboratory Fermentor #233395, Virtis Corporation, Gardiner, NY) containing 15 liters of 2xYT media (240 gm Difco Bactotryptone, 150 gm Bacto yeast extract, 2.5 gm sodium chloride, Difco Laboratories, Detroit, MI) and supplemented with 50 μM $Fe(II)SO_4$, 50 μM $ZnSO_4$, 50 μM Na_2S, 4 mM sodium thioglycolate, 100 μg/ml ampicillin, and 10 μg/ml tetracycline. The sealed flask was made anaerobic by gentle bubbling of nitrogen gas for 3 hours prior to cell inoculation. Anaerobicity was monitored by the presence of a small quantity of methylene blue (10 mg) which remains colorless in the absence of oxygen. After approximately 14 hours anaerobic culture at 37° C when the cell density had reached 0.05 OD (optical density) at 600 nm, 0.2% (w/v) D-(+) glucose was added. The culture was allowed to continue to 0.7 OD at 600 nm when 1 mM isopropyl-β-thiogalactopyranoside (IPTG) (Fisher Scientific, Pittsburgh, PA) was added to induce further expression of the *Clostridial* lysine 2,3-aminomutase gene. After 4 hours, the culture was cooled to 24° C and allowed to continue for an additional 12 hours before cell harvesting. Cells were harvested by concentration using tangential flow filtration (Pellicon System, Millipore Corporation, Bedford, MA) followed by centrifugation at 5,000 x g for 20 min. The cell pellets were snap frozen and stored in liquid nitrogen until used.

All subsequent operations were conducted in an anaerobic glove box (Coy Laboratory Products, Inc. Ann Arbor, MI). Cells (approximately 1-2 gms) were placed in 3 ml of 0.03 M sodium EPPS buffer (N-[2-hydroxyethylpiperazine-N'-[3-propanesulfonic acid]) pH 8 containing 0.1 mM L-α-lysine, 10 μM pyridoxal-5-phosphate, and 1 mM dithiothreitol (Sigma Chemical Co., St. Louis, MO). The cells were broken by sonication (Sonic Dismembrator #550, Fisher Scientific, Pittsburgh,

PA) using the microtip at a setting of 3 for five 20 sec. bursts with cooling on ice. The broken cells were centrifuged at 80,000 x gav for 30 min.

The supernatant was used to measure L-β-lysine formation according to the procedure of Ballinger et al. Biochemistry 31:10782 (1992). The procedure is based on the observation that radiolabeled L-α-lysine can be separated from radiolabeled L-β-lysine by paper electrophoresis in formic acid solution based on the difference in the pKa of the carboxyl group of each amino acid. The cell extract was incubated in 0.04 M EPPS pH 8 buffer containing 1 mM ferrous ammonium citrate, 0.5 mM pyridoxal 5-phosphate, and 20 mM dihydrolipoic acid for 4 hr. at 37° C. After the reductive incubation, the sample was diluted into 0.18 M EPPS pH 8 buffer containing 3 mM sodium dithionite, 18 μM S-adenosylmethionine, 44 mM C-14 labeled (#NEC280E-NEN Life Science Products, Boston, MA) and unlabeled L-α-lysine and incubated 4 min. at 37° C. The reaction was stopped by the addition of 0.2 M formic acid. The mixture was spotted onto chromatography paper (Whatman #3001917, Whatman, LTD, Maidstone, England), the amino acids separated by electrophoresis and radioactivity measured according to the published procedure. The cell extract exhibited lysine 2,3-aminomutase activity (4-5 units/mg protein). The specific activity of purified lysine 2,3-aminomutase from *Clostridium subterminale SB4* cells has been reported as 30-40 units/mg (Lieder et.al., Biochemistry 37:2578 (1998)). Thus lysine 2,3-aminomutase represents approximately 10-15% of total cellular protein in this expression system.

The recombinant produced lysine 2,3-aminomutase was purified according to the procedure of Moss and Frey, J. Biol. Chem. 265:18112 (1990) as modified by Petrovich et al., J. Biol. Chem. 226:7656 (1991), as previously discussed. The purified recombinant produced lysine 2,3-aminomutase had equivalent enzyme activity (34.5 ± 1.6 μmoles lysine min^{-1} mg^{-1} protein) to purified naturally produced *Clostridial* enzyme (Lieder et al., Biochemistry 37:2578 (1998).

All references cited above are hereby incorporated by reference.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed

embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.